ANALYSIS OF INTERCELLULAR WASH FLUIDS OF COTTON COTYLEDONS FOR THE PRESENCE OF PHYTOALEXIN-ELICITOR-ACTIVE OLIGOGALACTURONIDES

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LIST OF ABBREVIATIONS

Ac 44E	Acala 44E (susceptible cotton line)
Ara	arabinose
CDTA	*trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid hydrate
DOM	degree of methylesterification
dp	degree of polymerization
DP	degree of polymerization
DHC	2,7-dihydroxycadalene
fraction 375	oligogalacturonide mixture (dp = 8-16)
Fraction 375	oligogalacturonide mixture (dp = 8-16)
HM-pectins	high-methoxyl pectins
Im 216	immune 216 (resistant cotton line)
IWF	intercellular wash fluids
Gal	galactose
galA	galacturonic acid
GalA	galacturonic acid
HMC	2-hydroxy-7-methoxycadalene
LC	lacinilene C
LCME	lacinilene C 7-methyl ether
LM-pectins	low-methoxyl pectins
PGIP	polygalacturonase inhibitory proteins
PG	endopolygalacturonase
PGs	endopolygalacturonases

PL	endopectate lyase
PLs	endopectate lyases
PME	pectinesterase
PMEs	pectinesterases
PMG	endopolymethylgalacturonase
PMGs	endopolymethylgalacturonases
Rha	rhamnose
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
V_i	included volume
V_o	void volume
WbM (0.0)	Westburn M (0.0) (resistant)
WbM (4.0)	Westburn M (4.0) (suscecptible)
Xcm	*Xanthomonas campestris* pv. *malvacearum*
Xyl	xylose

CHAPTER I

INTRODUCTION

Carbohydrates are the building blocks of many of the structural polymers that give form to living cells and organisms. Compared to other biomolecules, carbohydrate molecules can be more structurally complex and exhibit a larger number of possible conformations and configurations. These molecules contain multiple hydroxy groups which allow them to exhibit a number of multiple linkages and display complex stereochemical structures (Stryer, 1989).

Carbohydrates, which play multiple roles in all forms of life, make up most of the organic matter on earth. Carbohydrates serve as energy stores, fuels, and metabolic intermediates. Starch in plants and glycogen in animals are polysaccharides that can be rapidly mobilized to yield glucose, a prime fuel for the generation of energy. Carbohydrates, such as ribose and deoxyribose, form part of the structural framework of DNA and RNA. The conformational flexibility of these sugar rings is important in the storage and expression of genetic information. Carbohydrates also serve as structural elements in the cell walls of plants and bacteria, the exoskeleton of arthropods, and the extracellular matrix of other animals. In fact, cellulose, the main constituent of plant cell walls, is the most abundant organic compound in the biosphere. Carbohydrates can also be linked to many proteins and lipids. For example, the sugar units of glycophorin give red cells a highly polar anionic coat (Stryer, 1989).

As discussed in a recent review by Albersheim and co-workers (Albersheim *et al.* 1992), carbohydrates play important roles in the interactions of living cells with one another as well as with their environment. These molecules are ideal ligands for precise

interactions with recognition sites on proteins. These protein sites can interact with the hydroxyl groups of carbohydrates. Between 10^4 and 10^5 different tetrasaccharides could be formed from interconnecting four glycosyl residues, with each tetrasaccharide adopting one or a few characteristic three-dimensional shapes and each requiring only a few specific enzymes for biosynthesis. This means that proteins distinguish among a large array of information-carrying carbohydrate molecules, information being transmitted to cells through carbohyrate-specific receptors.

The main topic of this investigation deals with carbohydrate molecules that may function as "elicitors" in the defense response of cotton, *Gossypium hirsutum*, against the bacterial blight pathogen, *Xanthomonas campestris* pv. *malvacearum*, *Xcm*. These "elicitors" are believed to be derived from the degradation of the pectic component of the plant cell wall as a result of bacterial infection. In these investigations, studies will be described on the following: 1) the biological activities of these carbohydrate molecules, 2) a stepwise analytical procedure that allows reliable quantitation of them from the cotton cotyledon intercellular spaces, and 3) the changes in the amount of these "elicitors" resulting from the interactions of the host cotton plant and the bacterial blight pathogen.

CHAPTER II

REVIEW OF LITERATURE

The Plant Cell Wall

Carbohydrates are the major component of plant cell walls. The plant cell wall is an elaborate extracellular matrix that encloses each cell in a plant. It is thick, strong, and rigid compared to the plasma membrane. Unfortunately, little is known about the three-dimensional organization of plant cell walls despite their importance in development and morphogenesis.

Each cell wall binds to that of its neighbors, cementing the cells together to form the intact plant. The walls of adjacent cells are glued together by a middle lamella, which is mainly made up of pectic polysaccharides and hemicelluloses, but its detailed composition is unknown. Pectic polysaccharide is the term given to plant cell-wall materials rich in galacturonic acid (galA) (Albersheim *et al.*, 1992). It includes the rhamnogalacturonan I, rhamnogalacturonan II, and the homogalacturonan regions (McNeil *et al.*, 1984). The homogalacturonan region is a linear polymer composed mostly of 1,4-α-D-galA residues (McNeil *et al.*, 1984; Albersheim *et al.*, 1992). The carboxyl groups of some of the galacturonosyl residues are methyl-esterified. When the degree of methylesterification (DOM) is zero, these polymers are known as pectic acids (Kertesz, 1951), and those with varying DOM are referred to as pectinic acids (BeMiller, 1986). Pectins are mixtures of polysaccharides that originate from plants, contain pectinic acids as major components, are water soluble, and are able to form gels under suitable conditions (BeMiller, 1986). Pectins are subdivided according to their DOM, a

DOM > 50% are referred to as high-methoxyl pectins (HM-pectins); those with DOM < 50% are referred to as low-methoxyl pectins (LM-pectins) (BeMiller, 1986). Pectic substances are mainly structural polysaccharides, occurring in the middle lamella and primary cell walls of higher plants. The homogalacturonans together with rhamnogalacturonans I and II comprise the bulk of the pectins of dicots (Varner and Lin, 1989). Degradation of middle-lamellar pectin results in tissue disintegration by cell-separation (maceration) (Rombouts and Pilnik, 1980). Thus, pectic substances are largely responsible for the integrity and coherence of plant tissues (Rombouts and Pilnik, 1980). The binding of Ca^{+2} to poly-D-galacturonate, the main backbone of pectin, has been investigated by equilibrium dialysis and circular dichroism (Morris *et al.*, 1982). Of the total stoichiometric requirement of bound Ca^{+2}, only 50% ± 5% is resistant to displacement by excess concentrations of univalent counterions. From these results, it was conlcuded that the co-operative binding of Ca^{+2} in polygalacturonate gels is through "egg box" complexes with the polysaccharide chains in analogous 2_1 conformations (Morris *et al.*, 1982). It is beleived that *in vivo* ionic binding (egg box structure) is likely to occur between two stretches of consecutive galA residues, with calcium ions acting as ionic bridges between negatively-charged galA residues. About 15-20 consecutive non-esterified galA residues are probably needed in each chain for stable complexes to be formed. Such stretches of galA residues occur in the homogalacturonan region and rhamnogalacturonan I; concerted binding will be interrupted in either case by methyl esterification and, in the case of rhamnogalacturonan I, by rhamnose residues. These "egg box" structures are likely to be of limited strength, but may occur frequently enough to give rise to a gel-like structure in the wall, similar to that found in isolated pectin and in fruit extracts used in jams and jellies (Brett and Waldron, 1990).

Mechanisms of Pathogen Virulence

Pathogens must possess the machinery required for growth in the environment of their hosts and must also be equipped to obtain nutrients and overcome host defenses in order to be successful in their attempt to colonize their host plant. Pathogens possess pathogenicity and virulence genes that are tightly regulated and generally expressed only when the parasite is growing in the host (Keen, 1992). Pathogenicity simply means the ability to cause disease; it is a qualitative term. Virulence is a quantitative measure of pathogenicity; the factors that are responsible for virulence are also important in the overall course of pathogenesis and symptoms development (Yoder, 1980). Pathogenicity genes, encoded for by the pathogens, are factors required for successful infection of host tissues (Dixon and Harrison, 1990). As described in a review article by Lamb and co-workers (1989), pathogenicity functions may include attachment to the plant surface, formation of fungal penetration factors, toxin production, or transfer and integration of T-DNA into the plant nuclear genome as is the case with *Agrobacterium tumefaciens*. As noted by Dixon and Harrison (1990), they may also include genes for the synthesis and extracellular transport of hydrolytic enzymes such as cutinase, polygalacturonase, and cellulase which may be involved in host cell wall degradation and therefore entry into host tissues.

Cutinase and its role in penetration of the cuticle has been studied extensively by Kolattukudy and co-workers (Kolattukudy, 1981; Koller, 1982). These workers concluded from their studies with *Fusarium solani f.* sp. *pisi* that the amount of cutinase released from the germinating spores was related to virulence of the isolate. Low virulence of an isolate on intact host pea plants could be increased by addition of cutinase and cell-wall-degrading enzymes to the conidial inoculum (Kolattukudy, 1981; Koller, 1982; Woloshuk and Kolattukudy, 1986).

The plant cell wall also serves as one of the major physical barriers to potential pathogens in their attempt to colonize the host plant. Since the plant cell-wall polysaccharides possess extreme structural complexity, pathogens encounter a large number of differently linked glycosyl residues plus noncarbohydrate substituents during the pathogens' attempts to penetrate and degrade plant cell walls. For a successful colonization, phytopathogenic bacteria and fungi must produce a wide array of enzymes capable of depolymerizing the polysaccharides of higher plant cell walls. Some of the enzymes produced by the pathogens are pectic enzymes. Pectic enzymes capable of cleaving the α-1,4-glycosidic bonds of homogalacturonans have been associated with the maceration of plant tissues (Byrde and Fielding, 1968; Mount *et al.*, 1970; Ulrich, 1975; Collmer and Keen, 1986; Benhamou *et al.*, 1990). The production of pectic enzymes is an important component of virulence in soft-rotting *Erwinia* spp. The most compelling evidence is from genetic experiments in which mutation of particular *pel* genes, genes that code for pectic enzymes, resulted in a marked decrease in virulence (Keen, 1992). Solubilization of the plant cell wall pectic polysaccharides, leads to products that are utilized as a source of nutrients by the pathogen (Kilgore and Starr, 1959; Collmer and Keen, 1986).

As discussed in a recent review article by Keen (1992), certain bacterial plant pathogens contain large gene clusters called *hrp* genes, which are required for growth and pathogenicity in plants. The *hrp* genes are also required to elicit hypersensitive defense reactions on incompatible host plants, but it is not yet clear whether this results from failure of *hrp* mutants to grow well in the plant or for more complex reasons. The biochemical functions of *hrp* genes are poorly understood, but some evidence suggests that they may be involved in the uptake and/or secretion of nutrients and other factors when bacteria enter host tissues.

Toxins and bacterial extracellular polysaccharides have also been implicated in the virulence of bacterial pathogens by genetic experiments. Recently, Kinscherf *et al.*

(1991) generated mutants of *Pseudomonas syringae* BR2 deficient in production of the toxin, tabtoxin, as well as the ability to produce disease symptoms on the host bean plants. The mutant strains multiplied as well on bean plants as the wild-type but did not produce disease symptoms. Clones that restored toxin production to both insertion and deletion mutants also restored the ability to cause disease (Kinscherf *et al.*, 1991). This is an important observation since it implies that host plants would be made resistant to disease if they could be made insensitive to the bacterial toxin (Keen, 1992).

Several plant pathogens cause imbalances in the levels of plant growth regulators. An example of work in this field comes from the group of Kosuge (Comai and Kosuge, 1982; Akiyoshi *et al.*, 1983) with *Pseudomonas syringae* pv. *savastanoi*, the causal agent of gall disease on olive and oleander plants. These bacteria multiply to large numbers in the favorable environment of the gall tissues, which are incited due to production of indole acetic acid by the pathogen. The indole acetic acid biosynthetic genes can therefore be considered as important bacterial virulence factors (Keen, 1992).

Specific Virulence Mechanisms

As discussed in a recent review by Keen (1992), specific virulence factors are produced by many pathogens that allow pathogenesis only on certain cultivars or species of plant. These factors consequently have importance in determining plant disease resistance and pathogen host range. For example, certain fungal pathogens produce low-molecular weight toxins that damage only specific cultivars of a host plant species. The pathogen only causes disease on these precise cultivars. Such host-selective toxins therefore constitute virulence mechanisms that exhibit a high degree of specificity since susceptible plant cultivars frequently harbor a single gene accounting for their toxin sensitivity.

Rhizobium spp. have been shown by mutation experiments to possess positive-acting genetic determinants required for nodulation of particular species and/or cultivars

of plants (Keen, 1992). Such determinants may also function to define the range of host species attacked by a pathogen. An example are the mutants of *Xanthomonas campestris* pv. *translucens* (Mellano and Cooksey, 1988) that were reduced in the number of grass host species they could attack. The bacterial strain employed normally attacked four different grass species, but the several mutants obtained had lost virulence on one or more of the plant species while remaining fully virulent on the others. Waney *et al.* (1991) confirmed these findings and were able to complement some of the mutations with cloned DNA from the wild-type strain. This should permit deduction of the various gene functions that compromise the resistance of particular plant species.

Swarup *et al.* (1991) showed that a cloned DNA fragment from *Xanthomonas citri* permitted another citrus pathogen, *Xanthomonas campestris* pv. *citrumelo*, to form canker-like lesions on citrus leaves which are normally unique to the disease caused by *Xanthomonas citri*. Thus, the cloned fragment appeared to confer a specific virulence factor associated with *Xanthomonas citri*. However, introduction of the same gene, called *pth*A, to other bacterial pathogens such as *Xanthomonas phaseoli*, and *Xanthomonas campestris* pv. *malvacearum* caused them to induce defensive reactions on their normal host plants. Another example involves *avr*Bs2, an avirulence gene cloned from *Xanthomonas campestris* pv. *vesicatoria* (Kearney and Staskawicz, 1990). When *avr*Bs2 was mutated in the bacterium, the resulting strain was reduced in virulence on pepper plants regardless of whether they harbored the cognate resistance gene, *Bs2*. Thus *Bs2* may target a bacterial metabolite specified by *avr*Bs2 that is important for bacterial virulence (Kearney and Staskawicz, 1990).

Pre-formed Barriers Employed by Plants Against Pathogens

The biochemical basis of defense mechanisms conferring disease resistance on plants is now under active investigation. Plant defense mechanisms are effective at different levels in host-parasite interactions and include preformed physical and chemical

barriers, such as the cuticle and the cell wall, as well as defenses triggered by the invading pathogens. The defenses triggered by the pathogens will be described later.

An impressive proof of the role of the cuticle in preventing ingress of certain pathogens is supplied by the work of Dickman *et al.* (1989). These workers transformed a plant pathogen, *Mycosphaerella*, which normally infects its host (papaya) through wounds in the cuticle/epidermis, with a cloned gene from *Fusarium*, which normally directly penetrates the cuticle and cell wall of its host plant (pea). *Mycosphaerella* transformants expressing the cutinase gene were able to directly penetrate the papaya fruit through intact cuticle.

In addition to such structural features, pre-formed chemical barriers may also be important in disease resistance. For example, a genetic experiment was utilized to test the role of the pre-formed steriodal glycoalkaloid tomatine in the resistance of green tomato fruits to infection by the fungus *Fusarium* (DeFago *et al.*, 1983). Mutant fungal strains were selected that grew in culture on high levels of tomatine and their virulence was also greater than the wild-type, tomatine-sensitive pathogen. This indicated that tomatine is an important resistance factor employed by tomato plants against *Fusarium* infection (DeFago *et al.*, 1983).

Pathogen-induced Defense Mechanisms Employed by Plants

Plants actively resist colonization by pathogens in a number of ways. One way is by the production of antimicrobial compounds, called phytoalexins, that kill the pathogen or restrict its spread. Other inducible defense mechanisms include reinforcements of the plant cell wall through the synthesis of lignin-like molecules and other phenylpropanoid materials and the accumulation of hydroxyproline-rich glycoproteins, which are incorporated into plant cell walls, causing the cell walls to be more resistant to microbial invasion. Another mechanism is the deposition of callose (ß-1,3-glucan) (Ebel, 1986).

Callose formation is believed to help in preventing the spread of the pathogen. Induced synthesis of hydrolytic enzymes such as ß-glucanase and chitinase by the plant may also serve to inhibit the pathogen, such as a fungus, by degrading their cell walls (Dixon and Harrison, 1990). All these defense mechanisms are produced locally in the vicinity of the infection site (Ebel, 1986).

As discussed by Dixon and Harrison (1990), rapid accumulation of phenolic material in plant cell walls to yield lignin is often associated with induced resistance. Lignin is a structurally complex phenolic polymer whose building blocks are hydroxycinnamyl alcohols. Lignin production is catalyzed by peroxidase through polymerization of phenylpropane alcohols. Induced lignification may be an important defense response, especially in certain monocots in which its rapid induction appears to be controlled by major resistance genes such as the *Sr5* gene for stem rust resistance in wheat. Lignification has been postulated to increase the resistance of plant cell walls to attack by hydrolytic enzymes of microbial origin (Bruce and West, 1989; Robertsen, 1986). This resistance is thought to be caused by covering and cross-linking of cell wall polysaccharides and proteins with lignin, which is very resistant to enzymic depolymerization (Hahn *et al.*, 1989a).

Hydroxyproline-rich glycoproteins accumulate in the cell walls of a number of plant species in response to microbial infection (Mazau and Esquerre-Tugaye, 1986) where they may act to strengthen cell walls. They may serve as a matrix for deposition of phenolic material such as lignin, or may possibly agglutinate pathogenic bacteria (Wilson and Fry, 1986; Varner and Lin, 1989). These proteins have the following characteristics: they are basic proteins with isoelectric points of about 10; they are rich in hydroxyproline residues that are typically glycosylated, and they have repeating structures that generally assume polyproline II helical structures that appear as flexous rods when examined by electron microscopy (Varner and Lin, 1989).

Induction of chitinase and 1,3-ß-D-glucanase activities appears to be closely related to induced defense in many plant species (Dixon and Harrison, 1990). Activities of both 1,3-ß-glucanases and chitinases appear to be induced at the same time by the same stimulus. These enzymes have the potential to hydrolyze fungal cell walls. Chitinase has no known substrates in plant tissues. Purified chitinase is antifungal, particularly against species in the genus *Trichoderma*, which have a high chitin content in their cell walls (Schlumbaum *et al.*, 1986). Glucanase can act as a synergist to increase antifungal activity of chitinase against a range of plant pathogenic fungi. Combinations of purified chitinase and 1,3-ß-glucanase from pea pods inhibited a number of fungi when protein preparations were added to paper discs placed on agar plates containing germinated fungal spores (Mauch *et al.*, 1988). Chitinase and ß-glucanase can also be induced in many plants by treatment with elicitors or ethylene (Dixon and Harrison, 1990). Ethylene-induced chitinase activity in bean leaves appears to be located primarily in the central vacuole of the cell, whereas glucanase is also found in the cell-wall space. These observations have led to the hypothesis that vacuolar chitinase and glucanase represent a final line of defense in microbial infection. On the other hand, cell wall-located glucanase could have a separate function in releasing "elicitor-active" molecules from invading pathogens and thereby activating "early" defense systems such as phytoalexin synthesis. In other plant systems, such as cucumber, oats, and carrots, chitinase may also be extracellular. Chitinase could also be involved in elicitor release, as chitin oligomers are active in eliciting phenolic synthesis in some systems. In cucumber, induction of chitinase in response to bacterial, fungal, and viral plant pathogens, as well as to abiotic stress, occurs both locally and systemically (Metraux and Boller, 1986). In addition to local defense responses, some plants are protected through induced systemic resistance, in which an initial infection at one site of the plant enhances resistance to subsequent infection in other parts.

As discussed by Ebel (1986), it is presently impossible to judge the relative importance of these general defense mechanisms in any host-plant interaction. Available evidence indicates that resistance to disease in most cases is the result of activation of more than one biochemical defense mechanism. It is also important to realize that resistance of a plant against a potential pathogen is not an invariable characteristic, but that the levels and patterns of the underlying defense reactions depend on both physiological and environmental conditions.

Pathogen-induced resistance in plants is rapid. Resistant potato leaf tissue (*Solanum tuberosum*) responds to infection by the late-blight fungus *Phytopthora infestans* very rapidly (Hahlbrock *et al.*, 1989). The earliest observable responses of immediately affected plant cells are callose deposition and the accumulation of phenolic compounds (*i.e.*, lignification) in the cell wall. Both responses are observed at about 2-3 h post-inoculation (Hahlbrock *et al.*, 1989). The accumulation of cell wall-associated phenolics at this early stage is an indication of hypersensitive cell death and is easily detected by autofluorescence of the cells under ultraviolet light.

The Hypersensitive Response

The hypersensitive response, HR, is a phenomenon commonly observed in plant-pathogen interactions. A plant, when challenged by a microbe that is either a species that is not a pathogen of that plant or is an incompatible race of a species that is pathogenic to other cultivars of that same plant, commonly exhibits a hypersensitive resistance response (Sequeira, 1983; Keen, 1992). The hypersensitive cell response is phenotypically observed as the rapid necrosis of one or a few plant cells at the site of attempted colonization. The necrosis is believed to result in processes that limit the spread of the pathogen (Sequeira, 1983; Keen, 1992). An example is the HR exhibited by bean pods to an incompatible isolate of *Pseudomonas syringae* (Webster and Sequeira, 1977). Inoculation of resistant bean pods with *Pseudomonas syringae* caused necrotic flecking

within the injected area by 24 h and these small lesions remained limited. By day 5, the population density of incompatible bacteria was about a one percent that of compatible ones. The appearance of the necrotic flecks was correlated with progressive decline of bacterial populations. Populations of incompatible bacteria within and between the necrotic flecks declined rapidly, indicating the accumulation of bacterial growth inhibitors. By day 3 substantial levels of the phytoalexin phaseollin had accumulated plus ethyl acetate soluble compounds that were found to be inhibitory towards the bacteria. The biochemical mechanism by which the hypersensitive cell death is triggered has not been established (Doares *et al.*, 1989a; Doares *et al.*, 1989b). As discussed in a recent review article by Keen (1992), while plant cell necrosis *per se* may contribute to resistance, the actual curtailment of the development and spread of the pathogen often occurs later. This evidence of resistance frequently correlates closely with the formation of phytoalexins or the erection of induced structural barriers such as lignin in the infection sites. Combination of defense responses may also act synergistically to contain an invading pathogen (Keen, 1992). The expression of many plant genes is activated during HR, including those encoding enzymes of the phenylpropanoid pathway, peroxidases, glucanases, and chitinases, as well as hydroxyproline-rich glycoproteins (Mazau and Esquerre-Tugaye, 1986; Keen, 1992; Reimers *et al.*, 1992).

Various molecules or preparations from either the pathogen or the host have been suggested as inducers of the HR. These may include cell-wall preparations and proteins. Examples are the cell-wall preparations from *Phytopthora infestans* that kill potato tuber tissue and a small peptide from intercellular fluids of fungal-infected tomato that kills tomato tissue (Doke and Tomiyama, 1980; De Wit *et al.*, 1985). Other examples are cell-wall fragments from sycamore and maize that caused cell death in cultured sycamore and maize cells (Doares *et al.*, 1989a). Plant cell death was monitored by inhibition of the ability of suspension cultured cells to incorporate [^{14}C]leucine (Doares *et al.*, 1989a). Another example is cryptogein, a 10 kD protein isolated from *Phytophthora cryptogea*

that elicited necrosis when applied to the petiole section of excised tobacco leaves (Milat *et al.*, 1991). Recently, Wei *et al.* (1992) isolated a proteinaceous elicitor of the HR from *Erwinia amylovora*, the causal agent in the bacterial blight of pear, apple, and other rosaceous plants. The elicitor, named harpin, is an acidic, heat-tolerant protein with an apparent molecular weight of 44 kD. The gene encoding harpin (*hrpN*) was located in the 40-kilobase *hrp* gene cluster of *Erwinia amylovora*, sequenced, and mutated with Tn5tac1. The *hrpN* mutants were not pathogenic to pear, did not elicit the HR, and did not produce harpin (Wei *et al.*, 1992).

Induced Phytoalexin Production as a Defense Mechanism

Plants respond to invasive microorganisms in a variety of ways. Among these ways, the synthesis and accumulation of toxic molecules called phytoalexins is observed in a number of plant-pathogen interactions (Hahn *et al.*, 1989a). The occurrence of phytoalexins in plant defense was implied by the classic work of Müller and Borger 50 years ago (Deverall, 1982). Phytoalexins are anti-microbial compounds of low molecular weight that are both synthesized by and accumulate in plants after the exposure of the plant to microorganisms (Paxton, 1981; Ebel, 1986). For a compound to be properly classified as a phytoalexin it should also satisfy the criteria of *de novo* synthesis in response to infection and accumulation to antimicrobial concentrations in the area of infection (Dixon and Harrison, 1990). Phytoalexins have broad-spectrum activity against many prokaryotic and eukaryotic organisms (Smith, 1982). Most phytoalexins are induced in plants by a range of metabolic or physical stresses in addition to infection (Dixon and Harrison, 1990).

Phytoalexins show the range and structural complexity typical of higher plant natural products. Their chemical natures are mostly phenylpropanoids, isoprenoids, and acetylenes (Stoessl, 1982). Phytoalexins are distributed among isoflavanoids, sesquiterpenes, diterpenes, polyacetylenes, dihydrophenanthrenes, stilbenes, and other

classes of compounds (Ebel, 1986). Phytoalexins have been isolated and characterized from members of a number of plant families, including *Leguminosae* (in which they are isoflavanoid derivatives or occasionally stilbenes), *Solanaceae* (sesquiterpenes and polyacetylenes), and *Umbelliferae* (furanocoumarins, isocoumarins, and chromones), just to mention a few (Dixon and Harrison, 1990). The discovery of phytoalexins in interactions between parasites and host plants has encouraged many investigators to beleive that these compounds are critical factors in regulating the interactions. Phytoalexin biosynthesis is strongly enhanced not only upon challenge of the plant tissues by pathogens but also following treatment with substances called "elicitors" (Ebel, 1986).

As discussed in the review by Ebel (1986), disease resistance in plants depends on multiple defense mechanisms for which the concept of coordinated defense has been proposed. The evidence that supports the role of phytoalexins in disease resistance has been discussed extensively. Phytoalexin production is not likely to be determined by the presence or absence of genetic information related to phytoalexin biosynthesis but rather by information specifying the expression of phytoalexin formation. Further, the ability to synthesize phytoalexins is not restricted to particular interaction between genotypes (*e.g.* race-specific vs nonhost resistance) but is more closely associated with morphologically similar types of response, *i.e.* there is close correlation between phytoalexin accumulation and HR. Most phytoalexins are present in very low quantities in healthy, unchallenged plants. Thus, the speed, magnitude, and site of accumulation of phytoalexins following penetration by the microorganism appear to determine disease resistance in some plant-microbe interactions. Experimental evidence that supports this conclusion has been obtained in a number of systems involving both fungi and bacteria (Bailey, 1974; Lyon and Wood, 1975). An example is the HR exhibited by hypocotyls of *Phaseolus vulgaris* (bean) when inoculated with spores of an incompatible race of *Colletotrichum lindemuthianum* (Bailey, 1974). Soon after exhibiting HR, four antifungal compounds accumulated in all infected tissues. In bean leaves inoculated with *Pseudomonas mors-*

prunorum, large amounts of the phytoalexins phaseollin and coumestrol accumulated soon after the hypersentive response became visible (Lyon and Wood, 1975).

A few studies have demonstrated that phytoalexins accumulate at the site of infection, and reach levels inhibitory to microbial growth quickly enough to play an active role in defense against further growth of the invading microorganism but may not be solely responsible for resistance (Hahn *et al.*, 1989b). A few studies have demonstrated that phytoalexin accumulation is limited, in incompatible (plant resistant) responses, to the immediate vicinity of the infection. Pierce and Essenberg (1987) found that in cotton leaves inoculated with *Xcm* the phytoalexins are concentrated in the fluorescent necrotic cells. Since the fluorescent cells are identical to the brown, necrotic cells, which are adjacent to the intercellular bacterial colonies of *Xcm*, the phytoalexins are concentrated near the bacterial colonies. These workers also found that by the time bacteriostasis is observed in inoculated resistant cotton plants, sesquiterpenoid phytoalexins have accumulated to concentrations that *in vitro* are completely inhibitory to *Xcm* (Essenberg *et al.*, 1985). In another study, Snyder and co-workers (1990), found that when sorghum leaves were inoculated with spores of the fungus *Colletotrichum graminicola*, phytoalexins were synthesized and accumulated in inclusions within the cells under attack. These observations are important since phytoalexins are toxic to both plant and microbial cells (Hahn *et al.*, 1989a; Essenberg *et al.*, unpublished observation).

As discussed in a review by Ebel (1986), the phytoalexin levels attained in plant-pathogen interactions are ultimately controlled by the rates of synthesis and degradation of phytoalexins as mediated by both host and pathogen interactions. Pathogen metabolism of phytoalexins may result in detoxification of these compounds and thus may allow successful pathogens to overcome the chemical barrier produced by these anti-microbial agents. Suppression of phytoalexin accumulation by pathogen-secreted phytoxins or other pathogen metabolites may be another way to hinder attainment of toxic phytoalexin levels.

Direct proof for a role for phytoalexins in disease resistance has been supplied by some genetic experiments utilizing strains of pathogen that vary in their virulence and ability to degrade a particular plant phytoalexin. Schafer *et al.* (1989) employed the phytoalexin detoxifying gene, *pda*, cloned from the fungal pathogen *Nectria haematococca*, which detoxifies the pea phytoalexin, pisatin. The *pda* gene encodes pisatin demethylase, which is a cytochrome P-450 monoxygenase that is mainly responsible for the detoxification of pisatin (VanEtten *et al.*, 1989a; Matthews and VanEtten, 1983). In one type of genetic experiment, the *pda* gene was transformed into and highly expressed in *Cochliobolus heterostrophus*, a fungal pathogen of maize but not of pea. Transformants contained at least as much *pda* as did wild-type *N. haematococca.* A significant growth of the transformed fungus occurred in pea plants and caused disease symptoms on pea (Schafer *et al.*, 1989). Thus, the ability to defeat a single component of pea defense allowed *Cochliobolus heterostrophus* to become a quasi pathogen. The antifungal activities of pisatin and the demethylated product, 6a-hydroxymaackiain were compared to test whether the initial metabolic reaction constitutes detoxification. Consistent with the hypothesis, several fungi (*e.g. Neurospora crassa, Penicillium expansum,* and *Helminthosporium turcicum*) were found to be substantially more sensitive to pisatin than 6a-hydroxymaackiain (VanEtten *et al.*, 1989a). Other examples are studies made with the phytoalexins medicarpin and maackiain, which are produced by a number of legumes such as chickpea. Field isolates of both *Nectria haematococca* and *Ascochyta rabiei* were tested for medicarpin and maackiain tolerance. The most virulent isolates were those that were able to metabolize the phytoalexins by at least one route (VanEtten *et al.*, 1989a; VanEtten *et al.*, 1989b). In the cases described above, the ability to degrade a host phytoalexin correlated with high virulence. In contrast, *Stemphylium botryosum* is sensitive to pisatin even though it can metabolize the phytoalexin (VanEtten *et al.*, 1989a; VanEtten *et al.*, 1989b). The ability to metabolize a phytoalexin therefore does not always confer tolerance to that compound. The fungus might still be sensitive

because it may still be intolerant of the product of pisatin metabolism or it may be metabolizing pisatin too slowly.

Role of Pectic Fragments of the Plant Cell Wall in the Response to Biological Stresses

Phytoalexin synthesis and accumulation is observed not only after microbial infection, but also upon treatment of plant tissues with elicitors (Davis *et al.*, 1986a; Sharp *et al.*, 1984a; Sharp *et al.*, 1984b; West *et al.*, 1985; Davis *et al.*, 1986b; Davis *et al.*, 1986c; Cheong *et al.*, 1991). The term "elicitor" was originally defined as any molecule or stimulus that induces phytoalexin synthesis in plants (Keen, 1975). The definition has now been extended to any group of molecules that stimulates any plant defense mechanism (*e.g.* lignification) (Hahn *et al.*, 1989b).

Molecules of microbial origin and cell-wall polysaccharides of plants have been implicated as "elicitors" of stress metabolite biosynthesis in higher plants responding to challenge by potentially pathogenic microorganisms (Bruce and West, 1982; Sharp *et al.*, 1984a; Sharp *et al.*, 1984b; West *et al.*, 1985; Bishop and Ryan, 1987; Bruce and West, 1989; Campbell and Ellis, 1992). Enzymes of microbial origin which catalyze the degradation of pectic polysaccharides of the plant cell wall have also been found to stimulate stress metabolite production in higher plants (Walker-Simmons, *et al.* 1984; Kurosaki *et al.*, 1985; Amin *et al.*, 1986; Davis *et al.*, 1984). This response is thought to be mediated by pectic fragments (*i.e.*, pectic oligomers). Pectic oligomers are released from the plant cell wall as a consequence of action by microbial enzymes (Davis *et al.*, 1986a; Davis *et al.*, 1986c; Forrest and Lyon, 1990).

"Elicitor-active" fragments of plant origin were first identified in the material released by partial acid hydrolysis of plant cell walls to produce oligomers (Hahn *et al.*, 1981; Nothnagel *et al.*, 1983; Moesta and West, 1985). Subsequently, it was shown that the same or very similar oligomers could be released by treatment of plant cell walls with

purified microbial pectic-degrading enzymes (Jin and West, 1984; West *et al.*, 1985; Davis *et al.*, 1986a; Davis *et al.*, 1986b; Moloshok and Ryan, 1989). These elicitor-active plant cell-wall fragments have been purified to apparent homogeneity and are mainly linear α-1,4-linked oligomers of D-galA (Nothnagel *et al.*, 1983; Jin and West, 1984; Davis *et al.*, 1986a). Oligogalacturonides that elicit maximum phytoalexin accumulation range in length between 10 and 13 galA residues and are active at micromolar concentrations (Nothnagel *et al.*, 1983; Jin and West, 1984; West *et al.*, 1985; Davis *et al.*, 1986a; Davis *et al.*, 1986b; Albersheim *et al.*, 1992).

These pectic elicitors have been tested in a wide range of assay systems, and depending on the size of the oligomers and plant species and tissue studied, have been shown to induce a spectrum of physiological effects. Linear α-1,4-linked oligogalacturonides of chain lengths varying between dp = 10-13 have been shown to induce phytoalexin accumulation and lignification (Nothnagel *et al.*, 1983; Jin and West, 1984; Davis *et al.*, 1986a; Robertsen, 1986; Bruce and West, 1989). In the castor bean bioassay for phytoalexin elicitation, the trideca-α-1,4-D-galacturonide showed the greatest elicitor activity on a weight basis (Jin and West, 1984; West *et al.*, 1985). Oligogalacturonides shorter than 8 glycosyl residues had little or no biological activity (Jin and West, 1984). In the soybean cotyledon bioassay for phytoalexin elicitation, the dodecagalacturonide was the most active component (Nothnagel *et al.*, 1983). Oligogalacturonides with dp = 11-13 have also been shown to be active in cotton (Pierce, unpublished observation). A purified pectic polysaccharide (*i.e.*, tomato leaf PIIF) from tomato leaves with mol wt ~5000 was active in eliciting phytoalexin accumulation in pea pod tissues (Walker-Simmons *et al.*, 1983). The mol wt was estimated by gel permeation chromatography on Sephadex G-50 with respect to known proteins. The mol wt was considered tentative since reliable carbohydrate standards in this mol wt range were not available (Ryan *et al.*, 1981). GalA (67% mol) was the major glycosyl residue of which 90% was methyl-esterified (Ryan *et al.*, 1981).

Elicitor-active oligogalacturonides were also obtained by treating sodium polypectate with an endopectate lyase (PL) (Davis *et al.*, 1986a; Davis *et al.*, 1986b; Komae *et al.*, 1990; Forrest and Lyon, 1990). In the soybean cotyledon assay, the fraction that contained the highest phytoalexin-elicitor activity contained predominantly a linear α-1,4-D-decagalacturonide with a single 4,5-unsaturated galA residue at the nonreducing terminus (Davis *et al.*, 1986a; Davis *et al.*, 1986b; Forrest and Lyon, 1990). The fraction with the second highest activity was found to contain predominantly the α-1,4-D-undecagalacturonide with a single 4,5-unsaturated galA residue at the nonreducing terminus (Davis *et al.*, 1986a; Davis *et al.*, 1986b). Only oligogalacturonides with a dp higher than nine were able to elicit phytoalexin synthesis in soybean cotyledons (Davis *et al.*, 1986a). In a similar set of experiments using the soybean cotyledon bioassay Forrest and Lyon (1990) also found that the deca- and undecagalacturonide products, derived by the action of endopectate lyases (PLs) from *Erwinia carotovora* and *Bacillus polymyxa* on potato cell walls, had the highest phytoalexin-elicitng activities. However, using the same soybean cotyledon bioassay, Komae and co-workers (1990) found that the hexagalacturonide was active, and it and the decagalacturonide, both derived by the action of endopectate lyase (PL) from *Erwinia carotovora* on water extracts of seeds from *Ficus awkeotsang* Makino, had the highest phytoalexin-eliciting activities. Komae and co-workers (1990) also reported that oligogalacturonides obtained from treatment of the highly methyl-esterified *awkeotsang* polygalacturonide (DOM = 62%) and low methyl-esterified apple pectin (DOM = ~30%) with the *Erwinia* endopectate lyase both elicited phytoalexin accumulation in the soybean cotyledon bioassay. The elicitor activity of *awkeotsang* oligogalacturonides appeared to be slightly higher than that of the apple oligogalacturonides. The authors attributed the difference to the different degrees of methyl esterification of oligogalacturonides or the difference in the proportion of the active molecules in the oligogalacturonide mixtures used for the assay (Komae *et al.*, 1990).

The chemical nature of pectic fragment elicitors and the structural features required for activity in the castor bean bioassay have been investigated by West and co-workers (1985). Full methyl esterification of a pooled mixture of elicitor-active oligogalacturonides (dp = 12-15), derived from treating polygalacturonic acid with the endopolygalacturonase (PG) from *Rhizopus stolonifer*, with diazomethane produced a derivative which was apparently less than 1/20 as active as the starting material or unmodified control (Jin and West, 1984; West *et al.*, 1985). In some cases, however, the results were not reproducible. In one instance, a methylated sample appeared to be as active as the non-derivatized sample (Jin and West, 1984). Oligogalacturonides obtained from deesterification of the methyl-esterified derivatives appeared to be four times more active than the starting material indicating that complete removal of the methyl groups was not attained. Two other experiments gave results that were very similar. Reduction of the reducing termini of a mixture of elicitor-active oligogalacturonides by sodium borohydride reduced their elicitor activity (West *et al.*, 1985). Jin and West (1984) have also shown that a variety of structurally different polyanionic polymers, such as chondroitin sulfate and hyaluronic acid, have no detectable activity. In the soybean cotyledon bioassay, Davis *et al.* (1986a) found that conversion of 90 to 95% of the galA residues to galactosyl residues abolished the elicitor activity of the decagalacturonide, demonstrating that at least some of the carboxylic acid groups of the galA residues are required for elicitor activity. From these results, it was concluded that the size of the oligomer, its polyanionic character, the presence of a reducing terminus, and perhaps other unidentified structural features of the galacturonosyl units are important structural features necessary for activity (West *et al.*, 1985).

In the soybean cotyledon bioassay, the decagalacturonide, obtained from treating polygalacturonic acid with PL, exhibited maximum elicitor activity at 5 μg/cotyledon (32 μ*M*) while the undecagalcturonide-rich fraction exhibited maximum elicitor activity at 6 μg/cotyledon (34 μ*M*) (Davis *et al.*, 1986a). The elicitor activity of the decagalactutonide

derived from polygalacturonic acid by the action of PL was 2-fold higher than that reported for a dodecagalacturonide obtained by partial acid hydrolysis of citrus pectin (Nothnagel *et al.*, 1983). The maximum amount of phytoalexin induced by the decagalacturonide was approximately half of the maximum amount induced by the ß-glucan elicitor and equivalent to that induced by PL (Davis *et al.*, 1986c). The soybean cotyledon bioassay is based on the following: ten cotyledons obtained from 8-day-old soybean seedlings are cut on the under surface, washed, and placed on a moist filter paper in petri dishes. The samples to be tested for elicitor activity are dissolved in water, and a 90-µL sample is applied to the cut surfaces of each of the ten cotyledons. The cotyledons with the test solutions on their cut surfaces are incubated in the dark for 20 h at 26°C and then transferred to 20 mL distilled water, and the absorbance at 286 nm of this 'wound-droplet solution' is measured. The absorbance at 286 nm is directly proportional to the amount of pterocarpan phytoalexins produced by the cotyledons (Hahn *et al.*, 1981). In the castor bean bioassay of Jin and West (1984), the concentration of individual oligogalacturonide tested for elicitor activity was 1.00 mg oligogalacturonide mL^{-1}. A dose response curve was performed for the elicitor activity of the fraction richest in the tridecagalacturonide, derived from treating polygalacturonic acid with the PG of *Rhizopus stolonifer*. The concentration required for 50% of the maximum response (ED_{50}) was 0.1 mg uronic acid mL^{-1} (43 µ*M*) (Jin and West, 1984). The castor bean bioassay is based on the following (Bruce and West, 1982; Jin and West, 1984): Ten seedlings with the radicles removed are cut along the plane dividing the cotyledons and placed, cut surface down, in a sterile Petri dish. The test solution (5 mL) is added to the petri dish and incubated for 10 to 12 h at 25°C in the dark. The pooled seedlings are homogenized, filtered, and centrifuged. A portion of the supernatant is removed from the floating lipid layer for the assay of casbene synthetase activity. One hundred µL of the supernatant is mixed with 50 µL water, and 250 µL Tris buffer. The reaction is initiated by the addition of 100 µL of 50 µ*M* [^{3}H]geranylgeranyl pyrophosphate (10 mCi $mmol^{-1}$) and allowed to

proceed for 30 min at 30°C before it is terminated by addition of ethanol:petroleum ether. The radioactivity associated with [^{3}H]casbene is extracted into petroleum ether and the extract is subjected to TLC on $AgNO_3$-impregnated plates. The radioactivity associated with casbene is measured in the 1-cm section at the $AgNO_3$ origin by liquid scintillation spectrometry (Jin and West, 1984).

Oligogalacturonides have been shown to activate other plant defense responses in addition to eliciting phytoalexin accumulation. The undecagalacturonide elicited lignification in cucumber cells (Robertsen, 1986). Oligogalacturonides with dp = 7-17 elicited lignification in castor bean suspension cultures (Bruce and West, 1989). Size-specific oligogalacturonides (dp = 10-15) enhanced the *in vitro* phosphorylation of a 34-kD protein associated with plasma membranes prepared from potato and tomato leaves (Farmer and Ryan, 1989; Farmer *et al.*, 1991). Shorter α-1,4-linked-galacturonides have been shown to induce the accumulation of proteinase inhibitors in tomato plants, another plant defense response (Bishop *et al.*, 1984; Bishop and Ryan, 1987; Thain *et al.*, 1990). In this case, molecules ranging in size from digalacturonide (dp = 2) to hexagalacturonide (dp = 6) are active (Bishop *et al.*, 1984; Bishop and Ryan, 1987).

Elicitation of necrosis in cowpea (*Vigna unguiculata*) pods by α-D-galacturonides has been observed (Cervone *et al.*, 1987a). Typically, circular portions (2-mm diameter) of epidermis were removed from each pod with a scalpel. The oligogalacturonide solution was applied in 6 μL of 50 m*M* acetate, pH 5.0. The appearance of the necrotic response was scored after 16 h of incubation and was characterized by a visible browning detected at the inoculation point. A mixture of α-D-oligogalacturonides with a dp higher than four, produced by partial digestion of sodium polypectate, elicited a necrotic response in *Vigna* pods that was indistinguishable from that elicited by *Aspergillus niger* PG, an enzyme that prefers deesterified pectic substrates. D-galA and the final PG degradation products of sodium polypectate, di- and monogalacturonic acid, did not elicit

a necrotic response. Oligosaccharides released from *Vigna* cell walls by partial digestion with PG were also active elicitors of the necrotic response (Cervone *et al.*, 1987a).

Fragments of the homogalacturonan region of the plant cell wall can also affect plant growth and development (Ryan and Farmer, 1991; Albersheim *et al.*, 1992). Pectic fragments, released from the walls of suspension cultured-sycamore cells by treatment with PG, inhibited formation of roots on tobacco thin-layer cultured cells grown on a root inducing medium containing indole-3-butyric acid and kinetin (Eberhard *et al.*, 1989). The pectic fragments induced polar tissue enlargement and the formation of flowers on tobacco thin-cell-layer explant bioassay. Pectic fragments isolated from the walls of suspension-cultured tobacco cells were as effective as those from the walls of sycamore cells in inducing *de novo* flower formation in the thin-cell-layer explant bioassay (Eberhard *et al.*, 1989). Oligogalacturonides with dp = 7-12 were able to induce a rapid and transient increase in ethylene biosynthesis when added to pear cells in suspension culture (Campbell and Labavitch, 1991). Oligogalacturonides have also been observed to cause rapid changes in plasma membrane functions (Ryan and Farmer, 1991; Thain *et al.*, 1990). Thain and co-workers observed rapid membrane depolarization when two size-ranges of pectic fragments (dp = 1-7 and dp = 10-20) were applied separately to tomato leaf cells. The depolarizations were rapid and reversible upon removal of the elicitors (Thain *et al.*, 1990).

The above observations point out that plants have the capacity to recognize biochemical stimuli originating from both the microorganism and from their own cell walls. The release of the phytoalexin-inducing oligogalacturonides *in vivo* at the onset of microbial attack is thought to be a mechanism by which the presence of the pathogen is signaled to the plant and the defense responses are activated (Cervone *et al.*, 1989). Recently, Cheong and Hahn (1991) have demonstrated the presence of a specific binding site for a fungal-derived hepta-ß-glucoside elicitor of phytoalexin accumulation in soybean microsomal membranes. Binding of the elicitor was saturable, reversible, and

had high affinity (apparent $K_d = 7.5 \times 10^{-10}$ *M*) comparable to the concentration of the hepta-ß-glucoside required for biological activity (Cheong and Hahn, 1991).

Classification of Pectic Enzymes

Pectic enzymes are clasified into two main groups, namely deesterifying enzymes (*i.e.*, pectinesterases) and chain-splitting enzymes (*i.e.*, depolymerases). The action pattern and specificity of each type of pectic enzyme is described below:

Pectinesterases

Pectinesterases (PMEs) (EC 3.1.1.11) are pectic enzymes produced by higher plants, numerous fungi, and some yeasts and bacteria (Papdiwal and Deshpande, 1983; Lin *et al.*, 1990; Rombouts and Pilnik, 1980). These enzymes deesterify pectin, producing methanol and pectic acid (Rombouts and Pilnik, 1980). Multiple molecular forms and isoenzymes of pectinesterase (PME) have been found in a number of fruits, as well as in certain fungi. The banana fruit produces two isoforms having pIs of 8.9 and 9.4 (Rombouts and Pilnik, 1980). PMEs from plants and fungi have a high specificity towards the methyl ester of pectinic acid (Lin *et al.*, 1990; Rombouts and Pilnik, 1980). The methyl ester of alginic acid (polymannuronic acid) and gum tragacanth are not attacked, and reduction of some methylgalacturonate to galactose (gal) residues causes a marked inhibition of enzyme activity (Rombouts and Pilnik, 1980; Rexova-Benkova and Markovic, 1976). The rates of hydrolysis of the ethyl ester of pectic acid by various plant and fungal enzymes is 10% to 20% of the rates of hydrolysis of the methyl ester (Rexova-Benkova and Markovic, 1976). Citrus PME deesterifies the ethyl ester of pectic acid at a rate 14% to 20% of that for the methyl esters (Rexova-Benkova and Markovic, 1976). Below a dp ~10, the rate of hydrolysis by orange PME decreases with decreasing chain length of the substrate and drops to zero for the trimethyl ester of the trimer (Rexova-Benkova and Markovic, 1976). The activity of plant PME is markedly influenced by

divalent and monovalent cations (Rombouts and Pilnik, 1980). An example is the alfalfa PME which is 30 times more active at pH 6 in the presence of Na^{+1} or Ca^{+2} (MacDonnell *et al.*, 1945).They are also competitively inhibited by pectate (end-product inhibition) (Rombouts and Pilnik, 1980). Oligogalacturonates with a dp = 8 and higher are effective inhibitors (Rombouts and Pilnik, 1980).

As discussed by Rexova-Benkova and Markovic (1976), the PME from the fungus *Fusarium oxysporum f.* sp. *vasinfectum* was found to be similar to the tomato pectinesterase. Both PMEs were found to affect highly esterified pectin. More than half of the enzymatic activity occurred at the reducing end of the molecule and the rest attacked a different locus or loci of the pectin chains. The action of PME does not proceed to complete deesterification of pectin but stops at a certain DOM. For tomato PME, the final value of 1.8% was found, as well as 0.4-0.6%. The action of *Pseudomonas prunicola* PME removed 75% of the methoxyl groups from different types of pectins, corresponding to final values of 0.6%-1.6% for methoxyl content. The action of *Coniothyrium diplodiella* PME removed 60% of the methoxyl group from pectin. All PMEs characterized from higher plants have pH optima between 7.0 and 9.0, while microbial PMEs have pH optima from 4.0 to 9.0.

Endopolygalacturonases

Endopolygalacturonases (PGs) (EC 3.2.1.15) are pectic enzymes produced by numerous plant-pathogenic and saprophytic bacteria and by fungi, and some yeasts (Keon and Waksman, 1990; Nasuno and Starr 1966; Solis *et al.*, 1990; Kurosaki *et al.*, 1985; Wijesundera *et al.*, 1989; Cervone *et al.*, 1987). They are also formed in higher plants, especially soft fruits (Bird *et al.*, 1988). A number of PGs, especially from fungi, have been purified (Keon and Waksman, 1990; Wijesundera *et al.*, 1989; Rombouts and Pilnik, 1980). Like the PMEs, multiple molecular forms and isoenzymes appear to be produced by many of the organisms tested. The majority of these enzymes have molecular weights

of about 36 kD and a glycoprotein nature has been established for many of them (Keon and Waksman, 1990; Rexova-Benkova and Markovic, 1976; Rombouts and Pilnik, 1980). The PG from *Aspergillus niger* consisted of a single peptide with an apparent mol wt of 34 kD (Cervone *et al.*, 1987b). As discussed by Rombouts and Pilnik (1980), PGs are, in general, specific for polygalacturonate. The kinetics and degree of hydrolysis of pectins decrease rapidly with increasing DOM. The activity on oligogalacturonides decreases with decreasing dp. The digalacturonide is not hydrolyzed and some of the enzymes do not attack the trimer. Random splitting of internal bonds of the D-galacturonan chain, which is catalyzed by PGs, results in a pronounced diminution in the viscosity of the substrate solution at a low degree of splitting of glycosidic bonds. Viscosimetry is a very sensitive assay method for PGs. With these enzymes, a 50% drop in specific viscosity of a pectate solution corresponds to hydrolysis of only a few percent of the glycosidic bonds.

The PG from the bacterium *Erwinia carotovora* is specific for non-methyl-esterified polygalacturonic acid (Nasuno and Starr, 1966). No products were observed when the enzyme was incubated with pectic acid that was 68% esterified (Nasuno and Starr, 1966). When incubated with polygalacturonic acid, the major end-products are monomeric and dimeric galacturonic acids. The dimeric galA is not hydrolyzed further. The hexamer is cleaved predominantly at the central bond, producing 2 molecules of the trimer. The pentamer is cleaved only into dimer and trimer. The PG from *Erwinia carotovora* attacks polygalacturonic acid by a random mechanism (Nasuno and Starr, 1966). The fungal PG from *Aspergillus niger* and *Sclerotinia sclerotiorum* consisted of numerous isoforms but a single PG is produced by *Colletotrichum lindemuthianum* (Keon and Waksman, 1990; Wijesundera *et al.*, 1989; Pressey, 1987; Rombouts and Pilnik, 1980).

Exopolygalacturonases

Exopolyagalacturonases (EC 3.2.1.67) are pectic enzymes found in higher plants, in the intestinal tracts of a number of insects, in fungi, and in some bacteria (Rombouts and Pilnik, 1980; Pressey, 1987; Hatanaka and Inamura, 1974). They are usually assayed by measuring reducing groups of liberated monomeric galA or more specifically by enzymic determination of galacturonate (Rombouts and Pilnik, 1980). In general, exopolygalacturonases from plants prefer moderately high (dp = 50) to high mol wt pectates (dp = 200), which they attack at the non-reducing end liberating monomeric galA. In general, these enzymes have optimum activities at pH of about 5 (Pressey, 1987; Rombouts and Pilnik, 1980). Plant exopolygalacturonases are stimulated by Ca^{+2} (Pressey, 1987, Rombouts and Pilnik, 1980). Hydrolysis of pectates does not proceed to completion, apparently because of structural irregularities of the substrates (Rombouts and Pilnik, 1980). Exopolygalacturonases of plant origin have been isolated. These include exopolygalacturonase from carrots, peaches, cucumbers, pears, apples, and oat seedlings (Rombouts and Pilnik, 1980). The exopolygalacturonase from the tomato fruit has mol wt ~47 kD (Pressey, 1987). Microbial exopolygalacturonases, however, have preference for oligogalacturonates. An example is the microbial exopolygalacturonase produced by *Aspergillus niger* which degraded oligogalacturonides with degrees of polymerization = 10, 4, 3, and 2 at least four times faster than sodium pectate (dp = 150) (Rombouts and Pilnik, 1980; Heinrichova and Rexova-Benkova, 1976). Divalent cations such as Ca^{+2}, Cu^{+2}, Mn^{+2},and Zn^{+2} did not affect the activity of the enzyme (Heinrichova and Rexova-Benkova, 1976). Mill (1966) isolated and purified an exopolygalacturonase from a mycelial extract of *Aspergillus niger*. The enzyme displayed its full activity only in the presence of Hg^{+2} ions. Other divalent cations such as Ca^{+2} and Mn^{+2} did not have effect on the activity of the enzyme. The enzyme hydrolyzed digalacturonic acid and trigalacturonic acid much more rapidly than pectic

acid and pectin was virtually resistant to attack (Mill, 1966). The exopolygalacturonase from *Coniothyrium diplodiella* was most active in the pH range of 4.0-4.5 (Endo, 1964). The exopolygalacturonase had little activity on pectin and pectinic acids which had been prepared from pectin by alkali-saponification method (Endo, 1964). When the hydrolysis of pectic acid by the enzyme was followed by measuring reducing groups released, pectic acid rapidly underwent a 70-80% hydrolysis within 5 h and was finally hydrolyzed completely to galA after 96 h. The exopolygalacturonase, however, had very little effect on pectin (Endo, 1964). Hasegawa and Nagel (1968) isolated an exopolygalacturonase from an aerobic *Bacillus* spp., known as isolate B. The pH optimum for the *Bacillus* spp. exopolygalacturonase was 6-6.5. The enzyme was highly specific for saturated oligogalacturonides and was completely inactive toward unsaturated oligogalacturonides. The *Bacillus* exopolygalacturonase preferentially attacked the short-chain uronides and hydrolyzed the terminal glycosidic bond from the nonreducing end of the molecule. The exopolygalacturonase activity was greatest with the trigalacturonic acid followed by tetramer, pentamer, dimer, and acid-soluble pectic acid (Hasegawa and Nagel, 1968). Hatanaka and Imamura (1974) have partially characterized the exopolygalacturonase produced by a *Pseudomonas* spp., known as strain P1, which was isolated from a soft-rotted radish. Incubation of the purified enzyme at pH 6.0 with pectic acid produced only digalacturonic acid. With trigalacturonic acid as substrate, digalacturonic acid was detected together with an equimolar amount of galA (Hatanaka and Imamura, 1974). In one case, Kimura and Mizushima (1973), partially purified an exopolygalacturonase from mycelial extracts of a strain of *Acrocylindrium*. The enzyme was most active at pH 4.5 and showed a higher affinity for polygalacturonic acid than for oligogalacturonic acids.

Endopectate Lyases

Endopectate lyases (PLs) (E.C. 4.2.2.2) are pectic enzymes that catalyze the cleavage of α-1,4-glycosidic bonds of D-galacturonans by the mechanism of ß-

elimination (Albersheim *et al.*, 1960). PLs are produced by various groups of bacteria and some plant pathogenic fungi (Garibaldi and Bateman, 1971; Collmer and Keen, 1986; De Lorenzo *et al.*, 1991; Davis *et al.*, 1986b). There are no reports of PL production by plants. The majority of these enzymes have very high optimum pH values and calcium ions or divalent ions are required for their activity (Weissbach and Hurwitz, 1959; Rexova-Benkova and Markovic, 1976; Collmer and Keen, 1986; De Lorenzo *et al.*, 1991). A common feature of *Erwinia* pectic enzymes is the presence of multiple isozymes for PL (Garibaldi and Bateman, 1971). An endopectate lyase purified from *Erwinia carotovora* exhibited optimum activity between pH 8.0 and 9.0 and was almost inactive at pH 7.0 or lower (De Lorenzo *et al.* 1991). The PL isozymes produced by *Erwinia chrysanthemi* have been extensively characterized (Garibaldi and Bateman, 1971; Collmer and Keen, 1986). Isozymes with alkaline, slightly alkaline and acidic isoelectric points have been resolved in culture supernatants by electrofocusing and chromatofocusing (Garibaldi and Bateman, 1971; Collmer and Keen, 1986). Studies by Forrest and Lyon (1990) with the PL from *Erwinia carotovora* suggested that methylation of citrus pectin reduced the ability of *E*rwinia *carotovora* PL to release wall fragments. The same workers compared the products released from different substrates by the PL from both *Bacillus polymyxa* and *Erwinia carotovora* and found that relative amounts of different oligogalacturonides released varied, especially the level of unsaturated tetramers (Forrest and Lyon, 1990).

The ß-eliminative action of PLs on their substrates results in the formation of products with double bonds between C-4 and C-5. Conjugation of the double bond with the carboxyl group at C-6 brings about absorption with a λ_{max} = 235 nm (Rombouts and Pilnik, 1980). PLs are therefore assayed most conveniently with an ultraviolet spectrophotometer, the molar extinction coefficient being 4600 cm^2 $mmol^{-1}$ (Rombouts and Pilnik, 1980). Another characteristic property of the products of ß-eliminative cleavage of pectic compounds is the reaction with thiobarbituric acid after cleavage with

periodate, giving rise to red condensation products having a maximum absorption at 547-550 nm. Both properties are used for the identification of ß-eliminative cleavage of pectic substances (Weissbach and Hurwitz, 1959; Rexova-Benkova and Markovic, 1976).

In general, the optimum substrates for PL are mostly deesterified galacturonans. However, Bateman (1966) and Sherwood (1966) have both described PLs from *Fusarium solani* f. *phaseoli* and *Rhizoctonia solani* that could split nonesterified substrates. These PLs also displayed higher activities with partly esterified substrates. For PLs from two *Arthrobacter* strains and from *Bacillus polymyxa*, the best substrates are pectates with DOM of 21, 44, and 26% esterification respectively (Rombouts and Pilnik, 1980). Different PLs differ in their action pattern toward polymeric and oligomeric substrates. A common feature of all these enzymes is the splitting of the glycosidic bonds of pectic acid and low-esterified D-galacturonans up to a certain degree of randomness, but the percentage of split glycosidic bonds corresponding to a 50% diminution in viscosity of the solution varies (Rombouts and Pilnik, 1980).

The highly substituted α-1,4-galacturonans such as gum tragacanth and gum karaya, are not degraded by PLs. The hydroxyl groups at C-2 and C-3, however, are not essential for the catalytic activity (Rombouts and Pilnik, 1980). Activity of PLs decreases with decreasing chain length of oligogalacturonate substrates. Most of the PLs described are capable of degrading trigalacturonate and unsaturated trigalacturonate as the lowest molecular-weight substrates (Rombouts and Pilnik, 1980).

Exopectate Lyases

Exopectate lyases (E.C. 4.2.2.9) are enzymes that liberate unsaturated dimers from the reducing end of pectate (Rombouts and Pilnik, 1980; Brooks *et al.*, 1990). As described by Rombouts and Pilnik, the exopectate lyases prefer high-molecular weight substrates. Exopectate lyase is the only known pectic depolymerase produced by *Clostridium multifermentans*, *Erwinia dissolvens*, and *Streptomyces nitrosporeus*. The

enzymes prefer pectate over pectins. Optimum pH for activity of most exopectate lyases are in the range of 8.0 to 9.5. The exopectate lyase produced by *Erwinia chrysanthemi* exhibited optimal activity between pH 7.0 and 8.0 when pectate was the substrate (Brooks *et al.*, 1990). Optimal activity was observed at pH 8.0 when citrus pectin was used as the substrate. The enzyme activity on pectate was enhanced by the addition of Na^{+1} and Ca^{+2} (Brooks *et al.*, 1990).

Endopectin Lyases

Endopectin lyases (E.C. 4.2.2.10) are the only depolymerases known to be specific for highly-esterified pectin and are known to be produced by fungi and two *Erwinia* species, strains of *Erwinia carotovora* and *Erwinia aroideae* (Rombouts and Pilnik, 1980). Endopectin lyases attack substrates at random, ultimately accumulating the lower unsaturated oligogalacturonate methyl esters, which resist further attack. Endopectin lyases isolated from various fungi and *Erwinia aroideae* have mol wt ~31 kD and have optimum pH ranging from 5.0-9.0 (Rombouts and Pilnik, 1980). The fungus *Colletotrichum lindemuthianum* produces two isoforms of endopectin lyase with pIs of 8.2 and 9.7 (Wijesundera *et al.*, 1989). The fungus *Sclerotina fructigena* produces two isoforms of endopectin lyase with a pH optima of 8.3 and 7.3 respectively (Byrde and Fielding, 1968). Unlike PLs, endopectin lyases have generally no absolute requirement for calcium ions although calcium may stimulate endopectin lyase activity. The stimulatory effect of $CaCl_2$ on endopectin lyase activity depends on the pH of the reaction mixture and the DOM of the substrate (Rombouts, 1972).

Role of Microbial Pectic Enzymes in Pathogenesis and in the Elicitation of the Defense Response

Microbial pectic enzymes may have at least two functions in plant tissues: first, the pectic enzymes are pathogenicity factors as they disrupt the structure of plant cell

walls, allowing microbial colonization of plant tissue and providing nourishment for the microorganism. On the other hand, the pectic enzymes are potential avirulence factors, since they may activate plant defense mechanisms by releasing cell-wall fragments that signal the plant to defend itself (Cervone *et al.*, 1989a). The possible dual functions of pectic enzymes are described below.

The contribution of pectic enzymes to plant pathogenesis has been reported by a number of investigators. PGs are the first detectable enzymes secreted by phytopathogenic microorganisms when grown *in vitro* on plant cell walls. PG is the first enzyme secreted by the fungus *Rhizopus stolonifer* during its attempts to colonize castor beans (Lee and West, 1981). PG is also the first detectable enzyme secreted by *Colletotrichum lindemuthianum* (Karr and Albersheim, 1972; Wijesundera *et al.*, 1989) and by *Fusarium oxysporum* f. sp. *lycopersici* (Jones *et al.*, 1972) when these pathogens are grown on isolated cell walls. Pre-treatment of plant cells with PG appears to facilitate attack of their substrates by the other microbe-secreted plant cell wall-degrading enzymes (Jones *et al.*, 1972; Karr and Albersheim, 1972). A number of investigators have correlated pectic enzyme activities to pathogenesis. Using an immunological screening method, Lei and co-workers (1985) identified a hybrid cosmid pSH711 from an *E. carotovora* genomic library that encodes PG activity but not PL activity. A cell extract of *E. coli* cells containing pSH711 was able to produce plant tissue maceration when spotted on carrot, potato, or turnip slices. In addition, the *E. coli* strain containing the plasmid was able to macerate carrot, potato, and turnip slices. Good correlation between endopectin lyase activity and the ability to macerate potato and apple tissue slices was found in fractions obtained from culture filtrates of the fungus *Sclerotinia fructigena* by ion-exchange chromatography (Byrde and Fielding, 1968). *Pseudomonas cepacia* and *Pseudomonas marginalis* both cause rotting of host tissues and readily produce pectic enzymes. While *P. cepacia* was observed to produced both PG and PL in culture, PG activity was the only pectic enzyme activity detected in rotting onion (Ulrich, 1975).

Mount and co-workers (1970) found a good correlation between tissue maceration and cellular death of potato tuber tissues in which the test solution contained PL with a pI of 9.2 from *Erwinia carotovora.* The action of the purified PL on sodium polypectate and induction of maceration and cellular death were directly correlated. Maceration and cellular death were measured by determining the increase in conductivity of the bathing solution or by the loss of ^{86}Rb from tissue which had been allowed to accumulate $^{86}RbCl$ prior to enzyme treatment. Also, the release of soluble unsaturated uronide products from potato discs by the PL was directly correlated with tissue maceration and cellular death (Mount *et al.*, 1970). Wijesundera and co-workers (1989) found a good correlation between PL activity and large-scale death of cells/collapse of tissue when bean hypocotyls were inoculated with the fungus *Colletotrichum lindemuthianum.*

The products of the enzymes are thought to be used as nourishment by the microbes. The catabolism of galA by *Erwinia carotovora* has been investigated (Kilgore and Starr, 1959). The initial steps in the catabolism of galA are isomerization to the keturonic analog (*i.e.*, 5-keto-L-galactonic acid) followed by reduction to the corresponding D-hexonic acid (*i.e.*, D-altronic acid). The initial steps are followed by a dehydration reaction leading to the formation of 2-keto-3-deoxy-D-gluconic acid and then phosphorylation by ATP to form the 2-keto-3-deoxy-6-phospho-D-gluconic acid. The phosphorylated product is subsequently converted to pyruvic acid and glyceraldehyde-3-phosphate. The catabolism of polygalacturonic acid by a *Pseudomonas* has been investigated by Preiss and Ashwell (1963). A polygalacturonate-degrading enzyme preparation has been partially purified from cell-free extracts of the pseudomonad and was shown to convert polygalacturonic acid to D-galA and 4-deoxy-L-threo-5-hexoseulose uronic acid, which are both subsequently converted to pyruvic acid and glyceraldehyde-3-phosphate through the 2-keto-3-deoxy-6-phospho-D-gluconic acid intermediate (Preiss and Ashwell, 1963).

As discussed earlier, a number of investigators have also shown that biologically-active oligomers of galA can be released from the plant cell wall by the action of two groups of microbial pectic enzymes, the PGs (Jin and West, 1984) and the PLs (Davis *et al.*, 1984; Davis *et al.*, 1986a; Davis *et al.*, 1986b; Forrest and Lyon, 1990). Several investigators have reported induction of a defense response after plant cells were challenged with pectic enzymes. Phytoalexin production in cultured carrot cells was induced when treated with purified pectic enzymes (Kurosaki *et al.*, 1985). Purified pectic enzymes from *Aspergillus niger* also elicited phytoalexin accumulation in suspension cultures of *Capsicum annuum* (Brooks *et al.*, 1986). Davis and co-workers (1984) detected heat-labile elicitors of pterocarpan phytoalexin accumulation in soybeans from culture filtrates of *Erwinia carotovora* grown on a defined medium containing citrus pectin as the sole carbon source. These heat-labile elicitors of phytoalexin biosynthesis co-purified with two PLs (Davis *et al.*, 1984). In another study, Bruce and West (1981) have shown that the PG isolated from culture filtrates of the fungus *Rhizopus stolonifer* was able to elicit the biosynthesis of an antifungal agent, casbene, in castor bean seedlings. The elicitor activity was lost upon heat inactivation of the enzyme (Bruce and West, 1981).

The dichotomy of the potential roles of pectic enzymes in plant-pathogen interactions raises the question of whether plants have a mechanism by which the balance between release of elicitor-active oligogalacturonides and the depolymerization of the active oligogalacturonides into inactive molecules could be altered to favor accumulation of elicitor-active molecules. Although synthesized and exported by the pathogen, pectic enzymes are not automatically expected to be active in the host since a variety of host plants have been reported to contain pectic enzyme inhibitors (Albersheim and Anderson, 1971; Cervone *et al.*, 1987b; Cervone *et al.*, 1989b). Cell walls of some plant tissues contain proteins, called polygalacturonase inhibitory protein (PGIP), that might counteract cell-wall degrading microorganisms by inhibiting pectic enzyme activities of

the pathogens. Proteinaceous inhibitors have been isolated and at least partially characterized from tissues or cell walls of bean, cucumber, alfalfa, pea, green pepper, tomato, apple, pear, peach, suspension-cultured sycamore cells, and citrus (Albersheim and Anderson, 1971; Collmer and Keen, 1986; Salvi *et al.*, 1990).

PGIPs inhibit fungal PGs but so far have not been found to inhibit fungal pectin or PLs, fungal exopolygalacturonases, or bacterial and plant PGs (Cervone *et al.*, 1990). PGIPs isolated from *Phaseolus vulgaris* inhibited all fungal PGs tested from *Aspergillus niger*, *Fusarium moniliforme*, and three races of *Colletotrichum lindemuthianum* but did not inhibit the activity of a bacterial PG from *Erwinia carotovora* and a bacterial PL from *Erwinia carotovora* (Cervone *et al.*, 1990). However, the PG from *Aspergillus niger* retained some residual activity even in the presence of excess of PGIP (Cervone *et al.*, 1990) thus suggesting that PGIP *in vivo* might generate oligosaccharides that are active in eliciting plant defense response thereby converting an essential pathogenicity factor into a trigger of plant defense responses. This is a hypothetical mechanism by which the activity of fungal PGs is modified by a constitutive plant protein such that the half-life of elicitor-active oligogalacturonides released by the PGs can be dramatically increased (Cervone *et al.*, 1989b).

Avirulence/Resistance Genes

As discussed in an article by Gabriel (Gabriel *et al.*, 1986), naturally occurring variation in the interactions of plant pathogens with their hosts is common, and many pathogenic taxa are subdivided into "races" based on their phenotypic reactions on a standard set of differential host varieties. Classical genetic studies of several pathogens and their hosts have shown that the race-specific reactions are controlled by avirulence genes (*avr*) in the parasites and resistance genes (R) in the host. Races of microbial pathovars are distinguished based on which avirulence genes they contain: the presence of an avirulence gene results in recognition, called an incompatible interaction, between

the potential pathogen and any line of the host plant which carries a corresponding resistance gene (gene-for-gene recognition).

As discussed by Harrison and Dixon (1990), a major phenotypic result of the action of host resistance genes is rapid response to avirulent pathogen races. At the cytological level, this is often associated with the localized host cell death of the hypersensitive response that restricts further pathogen development. At the molecular level, the host resistance genes are predicted to encode receptors that bind microbial determinants of avirulence (race-specific elicitors). The binding of the microbial determinants of avirulence to the receptors may lead to the activation of the defense response genes for the production of anti-microbial barriers. Shotgun cloning by function of resistance genes, using strategies analogous to those previously described for bacterial virulence and avirulence genes, is not yet feasible due to low plant transformation efficiencies and the large genome size of plants compared to bacteria. *Arabidopsis thaliana*, a small crucifer has lately received considerable attention as a model plant system. It is a self-pollinating diploid with a small genome size and is therefore better suited for genetic studies.

Avirulence genes are identified in a gene library from a widely avirulent strain by transfer of plasmids to a widely virulent strain and screening for acquisition of avirulence on specific host lines. Avirulence genes have been cloned by several laboratories from various *Pseudomonas syringae* pathovars and from some *Xanthomonas* spp. At least six avirulence genes that are clustered on a 90-kilobase plasmid in the bacterial blight pathogen, *Xcm* strain H, have been identified (De Feyter and Gabriel, 1991). Subcloning experiments resulted in the separation and partial localization of four avirulence genes, designated *avr*B4, *avr*b6, *avr*b7, and *avr*BIn. These four appeared to interact in a gene-for-gene manner with the corresponding resistance genes, but were not exclusive interactions. Subcloning also demonstrated the presence of two additional avirulence

genes, designated *avr*B101 and *avr*B102, on the *Xcm* strain H plasmid (De Feyter and Gabriel, 1991).

Bacterial Blight of Cotton

Bacterial blight of cotton has been reported in most cotton-producing areas in the last 70-80 years (Bird *et al.*, 1981). The disease is responsible for significant crop losses in cotton-growing areas of Asia and Africa. In the United States, the disease has been reduced to a minor one through a successful breeding program for stable, heritable resistance conditioned by several genes (Brinkerhoff *et al.*, 1984). Bacterial blight of cotton is caused by the pathogen *Xcm* (Brinkerhoff *et al.*, 1984). As described by Bird and co-workers (1981), *Xcm* is a gram-negative, rod-shaped bacterium which moves by means of a single polar flagellum. Colonies on nutrient agar vary from pale yellow to yellow, depending on the isolate, and are relatively slow-growing, convex, and smooth with entire margins. Optimal temperatures for growth in culture are between 25 and 30°C. Morgham and co-workers (1988) observed that susceptible cotton leaves of Ac 44, inoculated with the *Xcm* strain race 3, maintained on a light/dark cycle show collapsed areas by day 2 and by day 4 exhibited water-soaked edges around a dry center when the original inoculum concentration was 4×10^8 cfu mL^{-1}. Microscopically, granal and stromal membranes of the chloroplasts of *Xcm*-inoculated Ac 44 cotton cotyledons are damaged by day 3 and by day 4 the chloroplast had lost most of its membranes when the original inoculum concentration was ~10^6 bacteria mL^{-1} (Al-Mousawi *et al.*, 1982a). Degeneration of the mitochondria occurred after ultrastructural alteration in the chloroplasts and by day 6, the plasmalemma and the nucleus also showed some damage (Al-Mousawi *et al.*, 1982). Essenberg and co-workers (1979a) found that in leaves of susceptible cotton (*i.e.*, Ac 44), vacuum infiltrated with suspensions of a California field isolate of *Xcm* race 1, produced expanding water-soaked areas, whereas in the leaves of immune cotton line (*i.e.*, Im 216), small clusters of palisade cells became dark brown

(Essenberg *et al.*, 1979b). The number of water-soaked areas or brown cell clusters was proportional to the number of bacteria administered. A search for *Xcm* in the cotton leaves indicated that the brown cell clusters were the sites of bacterial growth, therefore, each brown cell cluster is formed in response to infection by a single bacterial cell or unseparated pair of cells (Essenberg *et al.*, 1979b).

Xanthomonads' Pectic Enzymes

The first demonstration of the ability of *Xanthomonas* spp. to produce pectic enzymes was by Burkholder and Starr when they observed that 38 of 77 xanthomonad cultures could liquefy a pectate gel (Starr and Nasuno, 1967). A few years later, Sabet and Dowson (1951) reported that four *Xanthomonas* spp. were able to liquefy pectate gel. They were *X. campestris*, *X. citri*, *X. malvacearum*, and *X. begoniae*. Relative to the soft-rot *Erwinias*, *Xanthomonas* spp. were able to liquefy pectate gel in a slower and less vigorous manner (Starr and Nasuno, 1967). It is suggested that the differences in the extent of liquefaction of the pectate gel by *Erwinia* and *Xanthomonas* spp. may be attributed to the nature and amount of the pectic enzymes they secrete (Sabet and Dowson, 1951). In the study by Starr and Nasuno (1967), of 19 nomenspecies of *Xanthomonas*, 10 were able to liquefy a nutrient pectate gel thus indicating hydrolytic/depolymerase pectic enzyme activity. None of the 19 nomenspecies produced either polygalacturonase or endopectin lyase. Of the 10 species that liquefied the pectate gel, 7 exhibited PL activities. The PL activities exhibited by the xanthomonads tested degraded polygalacturonic acid in a random manner with the major products being the unsaturated di- and trigalacturonides. Of the 19 nomenspecies of *Xanthomonas* studied, 17 exhibited detectable PME activities.

Pectic Enzymes of *Xanthomonas campestris* pv. *malvacearum*

The production of pectic enzymes by isolates of *Xcm* has been investigated. Abo-El-Dahab (1964) observed that 5 isolates of *Xcm* when grown in the presence of pectic substances were stimulated to produce pectic enzymes in culture. PME activity was found to be abundant in *Xcm* filtrates previously grown for 3 days on basal medium supplemented with pure pectin. Abo-El-Dahab (1964) also observed that *Xcm* culture filtrate preparations free from active cells adapted to pectin, calcium pectate, and galacturonic acid showed marked activity in disentegrating potato tuber discs after a 48 h incubation than culture filtrate preparations from active cells maintained on basal medium enriched with glucose. The above observation showed that culture filtrate preparations from pectin and calcium pectate adapted cells of *Xcm* have the ability to macerate potato tuber discs. Further, when washed cell suspensions of *Xcm* were sprayed over the surface of leaves of 6-week-old cotton seedlings, angular spots developed on cotton leaves within 12 days after inoculation with pectin-adapted cells of *Xcm*; plants inoculated with unadapted inoculum started to develop symptoms 21 days after inoculation (Abo-El-Dahab, 1964). Papdiwal and Deshpande (1983) also reported PME activity in culture filtrates of *Xcm* isolates cultivated for 24 h in a medium containing pectin. No PME activity was detected at 2, 4, 6, and 8 h (Papdiwal and Desphpande, 1983). Verma and Singh (1971) observed a reduction in the viscosity of a 1% solution of pectin when incubated with isolates of *Xcm*, indicating the presence of pectic depolymerase. Their attempts to show the presence of a lyase enzyme failed although PME activity was again demonstrated (Verma and Singh, 1971; Verma and Singh, 1975). Sattarova and co-workers (1972) also observed pectic enzyme activities of the depolymerase/hydrolytic-type when they observed reduction in the viscosity of 1% pectin solutions when different strains of *Xcm* , grown on Czapek's medium with 10% green mass of cotton stems and leaves, were added to the solution. The nature of the depolymerase/hydrolytic pectic

enzyme could not be ascertain since no further investigations were carried out. In one report, no pectic enzyme activities were observed from six races of *Xcm* when grown for 4 days in a medium that contained 1% pectin (Baldwin, 1970). Hopper and co-workers (1975) observed that a necrotic lesion on cotton leaves was produced by culture filtrates of *Xcm* and several other *Xanthomonas* spp. grown on autoclaved cotton leaf medium. The induction of necrosis caused by the *Xcm* culture filtrates was correlated with the activity of pectic enzymes. Pectic enzyme assays on the culture filtrates indicated that of a hydrolytic enzyme but not a lyase enzyme since no activity was detected by the thiobarbituric acid assay (Hopper *et al.*, 1975). In a study during the interaction of cotton and *Xcm*, Venere and co-workers (1984) observed evidence of hydrolytic/depolymerase pectic enzyme activities in both susceptible and resistant cotton lines. No PL activity was observed in either *Xcm*-inoculated susceptible or resistant cotton lines. Recently, Liao and co-workers (1992) found *pel* homologs (*pel* genes code for PLs) in *Xcm* genomic libraries when probed with a 1.2 kilobase genomic fragment cloned from *Pseudomonas viridiflava* that is known to encode PL. The above observations indicate that *Xcm* has the capability to produce various pectic enzymes. Further characterization of the pectic enzymes produced by *Xcm* is needed especially those produced *in planta* during its interactions with the host plant. Pectic enzymes produced by *Xcm* under culture conditions may not necessarily be induced or active during host-pathogen interactions. It is especially interesting to find out when the pectic enzymes are produced or in what order are they produced. Also it would be interesting to find out what factors induce pectic enzyme production by *Xcm* during host-pathogen interactions.

CHAPTER III

RATIONALE OF THE INVESTIGATION

The experiments described in the succeeding pages were directed towards the long term objective of understanding how the bacterial blight pathogen *Xcm* causes disease in genetically susceptible cotton lines and how the interaction is altered in genetically resistant cotton lines. More specifically, investigations described in this thesis were directed toward addressing the consequences of avirulence-/resistant-gene interactions in terms of any differences in amounts or character of oligogalacturonide fragments released from the cell wall that occur in compatible versus incompatible interactions. A hypothesis was tested; that is, oligogalacturonides are natural endogenous elicitors of the defense response in plants against pathogens. This focus was prompted because of a number of reports that link oligogalacturonides, which are active in a number of bioassays, to the induction of the defense response by the host plant against the invading pathogens and that such molecules may function as chemical messengers with specific regulatory functions. Interest in the oligogalacturonides is brought about because of insufficient understanding in the sequence of early events which ultimately leads to a successful resistance response. If oligogalacturonides indeed function as natural elicitors of the defense response of plants against pathogens, one would expect them to be present during resistant host plant-pathogen interactions and therefore one would expect to see differences in the oligogalacturonide-levels produced in incompatible versus compatible interactions. A possible scenario for the signal transduction pathway resulting from avr-/R-gene interactions is the elevation of the levels of oligogalacturonides during resistant response, therefore comparison of the amounts of these biologically-active molecules in compatible

versus incompatible interactions is required to acquire a better understanding of the biological role(s) of these molecules.

The possible role of oligogalacturonides in the resistance response of cotton plants against *Xcm* is supported by the following observations: 1) differences in the galA levels of probable plant origin observed in dialyzed intercellular wash fluids (IWF) prepared with water at 24 h and 48 h post-inoculation from *Xcm*-inoculated resistant cotton cotyledons compared to that in *Xcm*-inoculated susceptible cotton cotyledons showed that the galA level in the susceptible line increased ~1.8 relative to the mock-inoculated control, but the galA level in the resistant line became approximately 1/3 compared to that of the mock-inoculated control (Pierce, unpublished observation), 2) a rapid appearance of pectic enzyme activities was observed in extracts of *Xcm*-infected resistant cotton cotyledons compared to a later appearance of pectic enzyme activities in extracts of *Xcm*-infected susceptible cotton cotyledons (Venere *et al.*, 1984). The extracts of infected cotton tissue also showed a difference in substrate preference: those derived from infected susceptible cotton cotyledons exhibited higher pectic enzyme activities at pH 5 than at pH 7 and preferred polygalacturonic acid as substrate rather than citrus pectin; whereas, extracts derived from infected resistant cotyledons exhibited higher pectic enzyme activities at pH 7 than at pH 5 and preferred citrus pectin as substrate rather than polygalacturonic acid, 3) differences in a pectic enzyme activity measured from intercellular wash fluids (IWF) prepared from incompatible- and compatible-interactions showed that PG activity was observed only for susceptible cotton cotyledons but not resistant cotton cotyledons in IWF prepared 24 h post-inoculation with *Xcm* (Pierce, unpublished observation), and 4) oligogalacturonides show phytoalexin-elicitor activity in cotton cotyledons (Pierce *et al.*, 1987).

As a result of *avr*-/resistance-gene interactions based on the observations above, a possible scenario could be postulated; that is, pectic enzymes which degrade the pectic component of the cotton cell wall are not just virulence factors but may be regulated

differentially in incompatible versus compatible interactions. Because Venere and co-workers (1984) found maximal pectic enzyme activities in resistant interactions 4 h post-inoculation that declined rapidly thereafter, it is possible that large fragments are released from the cell walls of resistant cotyledons immediately after inoculation with *Xcm*, and the pectic enzyme responsible subsequently becomes inactivated; whereas, pectic enzyme activity in the susceptible cotyledons may appear later and not be subject to the same inactivation. The inactivation of pectic enzyme activity could be due to the production of pectic enzyme inhibitors such as PGIPs in the resistant cotton cotyledons as a result of bacterial infection. If indeed the hypothesis is correct, altered pectic enzyme activity may participate in eliciting phytoalexin production via the oligogalacturonide elicitors. The "premature" cessation of pectic enzyme activity in resistant leaves may result in longer half-lives for oligogalacturonides of approximately 12 residues in length, lengths which are known to elicit phytoalexins (Cervone, 1989b; Cervone *et al.*, 1990). Purified pectic enzymes can release oligogalacturonide elicitors from plant cell walls, but the digestion must be limited to be successful. If pectic enzyme activity is not inhibited in the susceptible interactions, it is reasonable to expect that the half-lives of the oligogalacturonides of approximately 12 residues in length will be shorter such that they are quickly converted to elicitor-inactive fragments once they are formed. Based on this scenario, one would expect an elevation of the concentration of elicitor-active oligogalacturonides in the incompatible interactions relative to those found in the compatible interactions.

The probability of our learning if the differences are consequences of race/cultivar specific interactions will be enhanced by extending the investigation to congenic susceptible and resistant cotton lines. Plant-derived oligogalacturonides which can be rinsed from the intercellular spaces of *Xcm*-infected cotton cotyledons from resistant and susceptible cotton lines were purified and analyzed in order to characterize whether oligogalacturonide fragments were generated and whether elicitor-active oligogalacturonides are among the products of such interactions. The presence of phytoalexin-elicitor-active

oligogalacturonides in cotton cotyledon intercellular spaces has never been demonstrated before.

CHAPTER IV

RESULTS AND DISCUSSION

Phytoalexin Elicitation with Oligogalacturonide Mixture

Resistant cotton leaves and leafy cotyledons accumulate three sesquiterpenoid phytoalexins plus the methyl ether of one of them when challenged with the bacterial blight pathogen, *Xanthomonas campestris* pv. *malvacearum* (Essenberg *et al.*, 1990). These phytoalexins are 2,7-dihydroxycadalene (DHC), lacinilene C (LC), and lacinilene C 7-methyl ether (LCME) (Essenberg *et al.*, 1982; Essenberg *et al.*, 1990; Essenberg and Pierce, 1992). The other compound accumulated is 2-hydroxy-7-methoxycadalene (HMC) (Essenberg *et al.*, 1982; Essenberg *et al.*, 1990). The term stress metabolites will be used to indicate the production of at least one of three sesquiterpenoid phytoalexins plus HMC. In the absence of HMC, the term phytoalexin will be used. The structures of the terpenoid phytoalexins plus HMC are shown in Figure 1.

As was discussed in the review of literature, phytoalexin synthesis and accumulation is observed not only after microbial infection, but also upon treatment of plant tissues with elicitors such as the oligogalacturonides. The ability of purified oligogalacturonides to elicit phytoalexin accumulation in cotton cotyledons has been investigated by Pierce and co-workers (Pierce *et al.*, 1986). They found that oligogalacturonides that are active in eliciting phytoalexin accumulation in other systems (*e. g.*, castor bean and soybean) are also active in a cotton cotyledon bioassay. They collected samples 24 and 67 h after treatment of resistant line OK 1.2 with 100 μg mL^{-1} and 500 μg mL^{-1} solutions of oligogalacturonides ranging in size from dp = 8-17. Samples harvested at 67 h post-infiltration were done during the morning of the third day after infiltration.

The 67 h harvest is approximately the same time that *Xcm*-inoculated cotyledons accumulate maximal levels of phytoalexins (Pierce and Essenberg, 1987). A 24 h harvest was done because if oligogalacturonides are indeed produced during *Xcm*-cotton interactions and that they elicit phytoalexin biosynthesis then it would take a shorter period of time for phytoalexins to accumulate if the oligogalacturonides are already present. The concentration of 100 μg mL-1 was used because it is the concentration of oligogalacturonide that is active in eliciting phytoalexin accumulation in other plants such as castor beans (Jin and West, 1984). There were several replicates per chain length of the oligogalacturonide. Oligogalacturonides with dp = 11-13 clearly showed activity although not all replicates had activity. From these results, oligogalacturonides were found to be active in eliciting phytoalexin biosynthesis in cotton just like in other systems (Jin and West, 1984; Nothnagel *et al.*, 1983).

The response in the above experiments was not uniformly active that is why a time course experiment with oligogalacturonide fraction 375 was done. The time of harvest may not had been right and since the response with 100 μg oligogalacturonide mL^{-1} was variable an oligogalacturonide concentration of 0.50 mg mL^{-1} (pH 7.0) was targeted. However, subsequent assay for galA of the 0.50 mg mL^{-1} solution of fraction 375 showed that it was only 0.45 mg mL^{-1}. Fraction 375 is a mixture of oligogalacturonides. Fraction 375 contained oligogalacturonides with dp = 8-16 (Maness *et al.*, 1991) and contained a total of 31.4% mole percent of undecagalacturonide, the dodecagalacturonide, and the tridecagalacturonide. The oligogalacturonide mixture was obtained by eluting the autoclave-hydrolysates of polygalacturonic acid from a DEAE Sepahadex A-25 column with 375 m*M* KCl, thus the name fraction 375 (Maness *et al.*, 1991). Fraction 375 was chosen since it contained the highest percentage of oligogalacturonides that were active in eliciting phytoalexins among the fractions available. Samples harvested for phytoalexin analysis were taken from the infiltrated area away from the site of needle prick and therefore shows no wounding so that the observed phytoalexin would simply be due to the

presence of oligogalacturonides. The needle prick damages 5 to 10 cells (Pierce, personal communication). The site of the needle prick was avoided because Pierce had observed a correlation between wounding and phytoalexin production (Pierce *et al.*, 1986). Control cotyledons were infiltrated with the same sterile water (pH 7.0) that was used to dissolve the oligogalacturonides. In general for each plant, one cotyledon was infiltrated with the oligogalacturonide solution and the other cotyledon was infiltrated with water.

Oligogalacturonide-treated cotyledons became limp after infiltration only in the infiltrated regions and were slow to transpire the infiltrated fluid. The water-treated cotyledons also exhibited limpness but not to the same extent, and they recovered turgidity within about one hour. The oligogalacturonide-treated cotyledons were still wilted when the first set of discs was harvested at about 1.5 h after infiltration. Successive harvests were made during the next three mornings (19 h, 42 h, and 66 h post-infiltration) prior to the beginning of the photoperiod and during the next two afternoons (30 h, and 54 h post-infiltration) in which the discs were harvested ~10 h into the 14-h photoperiod.

Macroscopic observations were made on both the oligogalacturonide-treated cotton cotyledons and the water-treated cotton cotyledons. In some of the cotyledons infiltrated with the oligomer solution, yellowish-brown color was observed in some areas of the infiltrated regions. These colors were observed mostly in the abaxial side and were termed "bronzing". Bronzing was observed as a collection of nearly microscopic spots on the cotyledons that exhibited the effect. Bronzing was first observed in the morning that followed the day of infiltration (~19 h post-infiltration). More bronzing effects were observed by the morning of the second day after infiltration (42 h post-infiltration). Close to 1/3 of the oligogalacturonide-infiltrated cotton cotyledons exhibited bronzing by that time.

Six harvests were made. Phytoalexins were extracted and quantitated from both the oligogalacturonide and water-treated cotyledons as described in the experimental section. Measurable phytoalexin accumulation was not observed from tissues harvested at about 1.5

h post-infiltration. The result indicates that oligogalacturonides did not activate the synthesis of phytoalexins immediately at the concentration used for this experiment. The harvests made at 19 h and 30 h post-inoculation also did not show measurable stress metabolite accumulation.

The 42-h samples accumulated measurable quantities of phytoalexins (Figures 2 and 3). The control cotyledons infiltrated with water and harvested at the same time did not show phytoalexin accumulation, thus proving that the components of the solution containing the oligogalacturonides were largely responsible for the accumulation of phytoalexins. The compounds elicited by the oligogalacturonide fraction were DHC (63%) and LC (37%). As was found by Pierce and co-workers (unpublished observation), the result indicates that oligogalacturonides are capable of eliciting phytoalexin accumulation in resistant OK 1.2 cotyledons. The harvest made in the afternoon (*i.e.*, 54 h post-infiltration) did not indicate measurable phytoalexin accumulation. The harvest made at 66 h post-inoculation also contained a measurable amount of phytoalexins (Figure 3). Both the 42 h and the 66 h samples were harvested before the photoperiod began. Low levels of phytoalexins were detected in the control cotyledons during the 66 h harvest. Figure 3 shows an apparent oscillation in the levels of phytoalexins detected. These oscillations have been observed before. Pierce and Essenberg (1987) found oscillations in the levels of DHC in OK 1.2 cotton cotyledons infected with *Xcm*. Significant levels of DHC were found in cotyledons harvested in the morning of the second day (before the start of the photoperiod) after the inoculation with *Xcm*. The levels found in the two successive harvests, both made during the photoperiod, had gone down. During the next harvest (*i.e.*, the morning of the third day and before the start of the photoperiod), the levels of DHC found were at their highest. The levels of DHC found in samples harvested in the afternoon of the third day after inoculation had decreased considerably (Pierce and Essenberg, 1987). Pierce (unpublished observation) observed oscillations in the levels of DHC and HMC when cotyledons were treated with the tridecagalacturonide (0.8 mg mL^{-}

^{1}). In one set of samples, measurable quantities of DHC and HMC accumulated at ~40 h post-infiltration. Samples harvested at ~30 h and ~48 h post-infiltration contained little or no DHC or HMC. The 62 h harvest contained maximal levels of both DHC and HMC while the next harvest point (~70 h) accumulated neglible levels of both stress metabolites. Peaks in response to *Xcm* and oligogalacturonides have been found to be sharp. Typically, one or two harvest times contained the bulk of the phytoalexins.

Because "bronzing" effects were exhibited by only some oligogalacturonide-infiltrated cotyledons, phytoalexin accumulation in individual discs was quantitated to see whether there was a correlation between "bronzing" and the amount of phytoalexin accumulation. Four discs harvested from oligogalacturonide-infiltrated cotyledon plus two discs harvested from water-infiltrated cotyledons from the 66 h harvest were analyzed for phytoalexins. Two of the four oligogalacturonide-treated discs exhibited the bronzing effect while the other two did not. All four oligogalacturonide-treated discs accumulated measurable amounts of phytoalexins. Only LC and DHC were observed in the samples. No correlation was observed between bronzing and the amount of phytoalexins accumulated. In one disc that did not exhibit the bronzing effect, 2.2 nmol cm^{-2} of phytoalexins were found. The other three discs, two of which exhibited bronzing, had phytoalexin levels of 0.17 nmol cm^{-2} (no bronzing), 0.33 nmol cm^{-2} (bronzing), and 0.26 nmol cm^{-2} (bronzing). The cotyledons infiltrated with water had phytoalexin levels of 0.06 nmol cm^{-2} and 0.20 nmol cm^{-2}.

Results from this experiment show that measurable quantities of phytoalexins could be observed ~42 h after infiltration with a mixture of oligogalacturonides at a concentration of 0.45 mg mL^{-1}. The experiment with the oligogalacturonide mixture (*i.e.*, fraction 375) was repeated but no response was obtained. It is possible that a concentration of ~0.50 mg oligogalacturonide mixture mL^{-1} may not be sufficient to provoke a uniform response so a dose response curve was done with a more purified oligogalacturonide (discussed next). Pierce (unpublished observation) has repeated the time course experiment with the

tridecagalacturonide and also found a peak from one of two replicates at ~47 h after infiltration. The results of the experiments with fraction 375 are comparable to that observed with *Xcm*-inoculated OK 1.2 cotyledons. Pierce and Essenberg (1987) found measurable phytoalexin accumulation in OK 1.2 ~45 h after inoculation with *Xcm*. Davis (unpublished observation) observed phytoalexin accumulation ~45 h post-inoculation in *Xcm*-inoculated cotyledons and did not observe measurable quantities of phytoalexins in samples harvested 20 h after post-inoculation. How rapidly the tissue collapses is most likely a factor on how soon phytoalexins accumulate. Pierce (unpublished observation) found a correlation between necrosis and phytoalexin accumulation. During the time course experiment with the tridecagalacturonide (Pierce, unpublished observation), cotyledons that exhibited necrosis at ~47 h post-infiltration contained substantial levels of phytoalexins while those that showed necrosis later displayed higher levels of phytoalexins at a later time (*i.e.*, ~66 h). Based on the results of the above experiments, it appears that phytoalexins accumulate to a maximal between 40 and 70 h after infiltration.

Stress Metabolite Elicitation with the Dodecagalacturonide

A dose response experiment with the dodecagalacturonide was performed because the experiment with the oligogalacturonide mixture was not quite reproducible. The highly-purified dodecagalacturonide was used at concentrations ranging from 9 $\mu g\ mL^{-1}$ to 0.546 $mg\ mL^{-1}$. The structure of the fully deesterified 1,4-α-dodecagalacturonide is shown in Figure 4. On average, the levels of stress metabolites, in $nmol\ cm^{-2}$, were derived from three replicates per dodecagalacturonide concentration, with each replicate representing ten discs (6.0 cm^2) harvested and pooled from ten different cotyledons. Cotyledons were infiltrated in the middle of the photoperiod and were harvested ~40 h after infiltration with the dodecagalacturonide solutions. Control cotyledons were infiltrated with the same water that was used to dissolve the dodecagalacturonide and were harvested at the same time the oligogalacturonide-infiltrated cotton cotyledons were harvested.

Macroscopic observations were made on four pots chosen at random the morning following infiltration. The four pots contained seventeen plants. Of the seventeen cotton plants observed, four cotyledons showed collapsed areas. The collapsed areas were on cotyledons infiltrated with the dodecagalacturonide solution. The size of the collapsed areas was less than 5% of the overall infiltrated area and was more visible from the top than from the underside of the cotyledons. Figure 5 shows two chromatograms of phytoalexins extracted from OK 1.2 cotyledons infiltrated with various concentrations of the dodecagalacturonide. Figure 6 and Table 1 show the levels of phytoalexins, expressed in nmol cm^{-2}, elicited in OK 1.2 when challenged with increasing concentrations of the dodecagalacturonide. No saturation was observed with the dodecagalacturonide (Figure 6). The shape of the curve is parabolic with an inflection point at around 0.273 mg mL^{-1}. Very little response was observed below a concentration of 0.034 mg dodecagalacturonide mL^{-1}. The dodecagalacturonide applied at 0.546 mg mL^{-1} elicited the highest amount of stress metabolites, 22% of the quantity elicited by *Xcm* at an inoculum concentration of 4.0 x 10^6 cfu mL^{-1} when the *Xcm*-infiltrated cotyledons were harvested at 64 h after inoculation. Of the stress metabolites elicited by the dodecagalacturonide solution at 0.546 mg mL^{-1}, 75% was HMC, 22% was DHC and 3% was LC (Table 1). No LCME was observed in any of the samples analyzed. LCME is usually observed in measurable quantities during the incompatible interactions between cotton and *Xcm* pathovars. In some cases, however, the levels of LCME measured were not as high as the levels of LC, DHC, and HMC found (Essenberg *et al.*, 1990). Pierce (unpublished observation) found lower levels of LCME compared to levels of DHC, HMC, and LC in cotton cotyledons treated with 0.80 mg mL^{-1} tridecagalacturonide.

Pierce (unpublished observation) found that the concentration of the purified dodecagalacturonide (*i.e.*, 0.546 mg mL^{-1}) which reproducibly elicited stress metabolite accumulation from the same experiment also caused some necrosis as determined by microscopic examination of Evans blue-stained samples: about 20% of the plant tissue

infiltrated with 0.546 mg mL^{-1} dodecagalacturonide showed scattered brown cells (Pierce, unpublished observation). Microscopic examination of necrosis was based on the ability of the cells of the dodecagalacturonide-infiltrated cotyledons to absorb Evans blue dye. The selective staining of damaged and/or dead cells with Evans blue depends upon exclusion of this pigment from living cells by the plasmalemma, whereas it passes through the damaged plasmalemma of dead cells and accumulates as a blue protoplasmic stain.

Calculations were made to evaluate whether the dodecagalacturonide had elicited levels of stress metabolites that are comparable to those elicited by *Xcm*. The levels of LC, DHC, and HMC in nmol cm^{-2} found in OK 1.2 after infiltration with the dodecagalacturonide were converted to nmol stress metabolites g^{-1} dry wt. This was done by dividing the amount of stress metabolites found in nmol cm^{-2} by the dry weight of 1 cm^2 of the cotyledon (*i.e.*, 0.003 g). At 40 h post-infiltration, the dodecagalacturonide elicited 1.60 nmol DHC cm^{-2} (5.3×10^2 nmol g^{-1} dry wt), 0.23 nmol cm^{-2} LC (77 nmol g^{-1} dry wt), and 5.5 nmol cm^{-2} HMC (1.8×10^3 nmol g^{-1} dry wt) when applied at a concentration of 0.546 mg mL^{-1} (Table 1). The total stress metabolites elicited by the dodecagalacturonides applied at 0.546 mg mL^{-1} was ~2400 nmol g^{-1} dry wt which compared favorably with the total DHC, HMC, and LC elicited (*i.e.*, ~2930 nmol g^{-1} dry wt) by Im 216 at 70 h post-inoculation after challenged with *Xcm* (Essenberg *et al.*, 1990).

Stress Metabolite Elicitation with the Tridecagalacturonide

The ability of the tridecagalacturonide to elicit stress metabolite synthesis was also tested. The reasons for doing the experiment were the following: 1) to compare the amounts of stress metabolites elicited by the tridecagalacturonide to those elicited by the dodecagalacturonide, 2) to compare the amounts of stress metabolites elicited in a highly resistant cotton (*i.e.*, OK 1.2) vs. amounts elicited in a susceptible cotton line (*i.e.*, Ac 44E), and 3) to evaluate the shape of the dose-response curve at a higher concentration since the dose had not been saturating in the dodecagalacturonide experiment. Four

concentrations of the tridecagalacturonide were tested ranging from 0.25 mg mL^{-1} to 1.50 mg mL^{-1}. Cotyledons were harvested at two times, 22 h and 40 h post-infiltration. The 40 h harvest was made because of the results from previous experiments which indicated that maximal levels of phytoalexins accumulate between 40 h and 70 h post-infiltration. A 22 h harvest was also done because some of the concentrations of the tridecagalacturonide used were high enough that there is a possibilty that phytoalexins may accumulate early and just in case the time course experiment was not indicative of the right time to collect samples. The 22 h harvests were made ~ 5.5 h into the photoperiod while the 40 h harvests were made ~1.5 h before the start of the photoperiod. Each sample represented three discs, one each from three different cotyledons, in OK 1.2, and five discs, taken from five different cotyledons, in Ac 44E. All concentrations of the tridecagalacturonide elicited stress metabolite biosynthesis in both OK 1.2 and in Ac 44E (Tables 2 and 3). Sample chromatograms are shown in Figure 7. Tridecagalacturonide-treated cotton cotyledons harvested at 22 h post-infiltration elicited significant amount of stress metabolites. Higher levels of stress metabolites were observed in Ac 44E than in OK 1.2 during the 22 h harvest (Table 2). However, OK 1.2 cotyledons harvested at 40 h post-infiltration elicited higher levels of stress metabolites than the Ac 44E cotyledons harvested at 40 h (Table 3). An apparent saturation of the receptors was observed in OK 1.2 when samples were harvested 40 h after infiltration. The shape of the curve is sigmoidal. The dose response curve for Ac 44E at 40 h post-infiltration was a shallow parabola (Figure 8). Higher stress metabolite levels were found in both OK 1.2 and Ac 44E in the 40 h harvest than in the 22 h harvest. Higher levels of stress metabolites were elicited by the tridecagalacturonide at 1.00 mg mL^{-1} than at 1.50 mg mL^{-1} in OK 1.2 cotyledons (see Figure 8 and Tables 2 and 3). The tridecagalacturonide applied at 1.50 mg mL^{-1} elicited the most stress metabolites in Ac 44E at 40 h post-infiltration (Figure 9 and Table 3). When applied at a concentration of 1.50 mg mL^{-1} in Ac 44E, the tridecagalacturonide elicited only ~37% of the stress metabolite levels elicited by the tridecagalacturonide in OK 1.2 cotyledons at a

concentration of 1.00 mg mL^{-1} at 40 h post-infiltration. Of the stress metabolites elicited in Ac 44E harvested at 40 h post-infiltration at a concentration of 1.50 mg mL^{-1}, 65% was HMC, 25% was DHC, and 10% was LC (Table 3). Of the stress metabolites elicited in OK 1.2 when samples were harvested 40 h after infiltration at a tridecagalacturonide concentration of 1.00 mg mL^{-1}, 45% was DHC, 28% was HMC, and 27% was LC (Table 3). No LCME was detected in any of the samples analyzed.

The results of the three experiments conducted with the oligogalacturonides demonstrate that phytoalexin accumulation in cotton cotyledons can be triggered not only by *Xcm* but also by plant-derived components such as oligomers of galacturonic acid. The apparent oscillations obeserved in *Xcm*-inoculated cotyledons are also observed in the oligogalacturonide-treated cotyledons. At higher doses of oligogalacturonide used, measurable quantities of phytoalexins were elicited earlier. However, the levels of stress metabolites were greater at ~40 h post-infiltration than those observed at 22 h. As was the case with *Xcm*-inoculated cotyledons, it takes ~40 h in order to see measurable phytoalexin accumulation after cotyledons are infiltrated with oligogalacturonides if the doses used were not high enough.

It is not known how oligomers of galacturonic acid trigger the events that ultimately lead to accumulation of phytoalexins. Presumably, these molecules could bind to receptors in the host plant and this binding to the receptor(s) could start the cascade of events that may eventually lead to the synthesis of phytoalexins. The presence of a receptor for the dodecagalacturonide in soybean suspension cultures was indirectly shown by Horn and co-workers (1989). They showed that fluorescein-labeled and [^{125}I]-dodecagalacturonide were internalized by soybean suspension cultures. Within a few hours, virtually all of the elicitor was concentrated in the major vacuole. Endocytosis was highly temperature-dependent. Further, when 1 m*M* KCN was added to the cell suspension cultures to block any energy-dependent processes, no internalization of the dodecagalacturonide was detected. Furthermore, when a 10-fold excess of unlabeled dodecagalacturonide was

added to compete for specific receptors on the plant surface, the rate of uptake of labeled dodecagalacturonide was reduced at least 10-fold. Non-specific proteins (*e.g.*, ovomucoid) neither bound to the surface nor were they internalized in parallel assays.

If indeed these phytoalexin-elicitor-active oligogalacturonides are the actual signals that bind to the receptor(s), the binding site(s) of such receptors should be large enough to accomodate the relatively large size of these signal molecules. In a study made by Sharon and co-workers (1982) on the combining sites of seven IgM, and five IgA hybridoma antibodies specific for linear α-1,6-linked dextrans, these workers found that all 12 antibodies tested were able to bind linear determinants in the interior of the dextran molecule. Furthermore, six of twelve antibodies tested had specific sites complementary to six α-1,6-linked glucose residues (dp = 6) while the remaining six had specific sites complementary to seven α-1,6-linked glucose residues (dp = 7). Thus, the binding sites of these antidextran antibodies were about 25 Å long. An interesting comparison here is with lysozyme, which also has space for a hexasaccharide in its active-site cleft (Kabat, 1978). The hexasaccharide that lysozyme recognizes is made up of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues. With these results in mind, it is possible to envision the start of the signal transduction process for phytoalexin elicitation when two such receptors that could accomodate six or seven sugar residues could come together to bind either the dodecagalacturonide or the tridecagalacturonide.

Nakahara and Ogawa (1990) have recently synthesized the α-1,4-dodecagalacturonide through a highly stereoselective glycosylation that involved glycosyl fluorides as the donors and oxidation of the twelve primary hydroxyl groups in the α-1,4-galactododecaoside derivative. The biological activity of the synthetically-derived dodecagalacturonide has not been reported.

Because oligogalacturonides are active in eliciting stress metabolite accumulation not only in cotton cotyledons but also in a number of other systems (Nothnagel *et al.*, 1982; Jin and West, 1984), a study was undertaken in order to find out if these

phytoalexin-elicitor-active oligogalacturonides are actually produced *in vivo* when cotyledons are challenged with the bacterial blight pathogen, *Xanthomonas campestris* pv. *malvacearum*. As was mentioned in the last chapter, the presence *in planta* of phytoalexin-elicitor-active oligogalacturonides has not been demonstrated before. Analytical procedures were devised toward that goal.

Sugar Composition of IWF Collected from *Xcm*-inoculated Resistant (OK 1.2)and Susceptible (Ac 44E) Cotton Lines at Various Times after Inoculation

The role of oligogalacturonides in the defense response of cotton against the bacterial blight pathogen, *Xcm*, is the main focus of this investigation. With this in mind, the following questions were asked: a) are oligogalacturonides produced during the attempted invasion of cotton cotyledons by the bacterial blight pathogen? b) if so, are phytoalexin-elicitor-active oligogalacturonides among the products of such interactions? and c) what time is suitable for analyzing for the presence of these elicitor-active oligogalacturonides?

A sugar composition analysis of IWF was performed to help determine the optimal time to analyze for the presence of the oligogalacturonides. Two-week-old OK 1.2 cotyledons (~20 cm^2) were inoculated with *Xcm* (1.71 x 10^8 cfu mL^{-1}) and IWF were collected at several times using water as extractant. There were two replicates per sampling time. On average, each replicate represented an area equivalent to 93 cm^2 of plant material taken from 8 different cotyledons. The IWF were dialyzed (1000 mol wt cut-off) for ~16 h, which removed a fraction (~40%) of the monomeric galA (Mort *et al.*, 1991). Oligomers with dp > 2 are practically retained inside the dialysis membrane over a 24 h period when dialyzed in a 1000 mol wt cut-off membrane (Mort *et al.*, 1991). GalA as well as other sugars were quantitated by gas chromatography. The result of the time course showing the galA levels in nmol cm^{-2} found in IWF from *Xcm*-inoculated OK 1.2

cotyledons is shown in Figure 9. Maximal accumulation of galA was observed at 8 h post-inoculation. The levels found during the succeeding harvests were lower. The galA levels measured in the time course are indicative of both the extractable polymeric and oligomeric galA plus ~60% of the monomeric galA. Results of the sugar composition analysis show that about 8 h post-inoculation would possibly be a good time to analyze for the presence of phytoalexin-elicitor-active oligogalacturonides (*i.e.*, dp = 11-13) since the galA content of the IWF was maximal at that point. At 8 h post-inoculation, the galA content of the IWF collected from *Xcm*-inoculated cotyledons had increased by ~4.5 times relative to the IWF prepared at 0.5 h post-inoculation (Table 4). Pierce (unpublished observation) had also observed maximal levels of galA at 8 h post-inoculation which decreased thereafter when IWF were prepared from *Xcm*-infected OK 1.2 cotyledons. At 8 h post-inoculation, other sugar levels had not increased as much as the galA levels although arabinose (ara), galactose (gal), and rhamnose (rha) levels seemed to show an increasing tendency over a period of time (Table 4). Ara, gal, and rha levels at 24 h post-inoculation increased ~2 times relative to the IWF collected at 0.5 h. Pierce (unpublished observation) also observed an increase in the ara and gal levels (*i.e.*, 4- to 5-fold increase) over a 24 h period in the IWF collected from OK 1.2 cotyledons inoculated with *Xcm*. The rha levels, at 24 h post-inoculation, also increased ~2 times when compared to the rha levels observed from *Xcm*-inoculated cotyledons when the IWF was prepared at 0.5 h after infiltration. The xylose levels did not significantly change over a 24-h period. The glucose, glucuronic acid, and mannose levels were not tabulated because the amounts of these sugars were influenced by the presence of the bacterial extracellular polysaccharide (Pierce *et al.*, 1992). The extracellular polysaccharide elaborated by *Xcm* was not removed by centrifugation.

A sugar composition analysis of IWF collected from Ac 44E was performed to see the trend in a susceptible cotton line. Fourteen-day-old cotyledons were infiltrated with *Xcm* (1.66×10^8 cfu mL^{-1}) and IWF were collected at several times after inoculation using water as the extractant. There were two replicates per time. On average, each replicate

represented ~130 cm^2 of plant material taken from ~10 cotyledons. GalA levels as well as other sugars were processed and quantitated by the same method as that described for OK 1.2 earlier. The galA levels in the IWF collected from the susceptible cotton line infected with *Xcm* showed a significant increase in galA levels by 8 h after inoculation. The levels of galA observed in the IWF collected from the susceptible interactions at 12 h, 16 h, 20 h, and 24 h post-inoculation were not significantly different with that observed at 8 h post-inoculation. The galA level at 8 h post-inoculation was ~4 times that at 1 h post-inoculation (Figure 9). Only galA increased significantly by 8 h post-inoculation and unlike in the resistant interactions (*i.e.*, OK 1.2 + *Xcm*), the ara and gal levels were not substantially higher at 24 h post-inoculation but they did show a transient increase that peaked at 20 h (Figure 9). Rha and xyl levels did not change significantly over the 24 h period (Table 4).

The apparent increasing trend in the levels of ara and gal observed in the IWF prepared from the incompatible interactions might be an indication of a resistant response of OK 1.2 cotyledons against *Xcm*. Recently, Pierce and co-workers (1992) have detected more glucuronic acid than was expected from the exopolysaccharide produced by *Xcm* in the resistant cotton cotyledons during the early stages of infection. At the same time, a 4- to 5-fold increase in the ara and gal levels were observed only in the IWF prepared from *Xcm*-infected resistant cotyledons. They attributed the increases in the levels of glucuronic acid, arabinose, and galactose in samples from inoculated resistant cotton to the accumulation of an arabinogalactan-protein (a protein that contains variable but significant amount of glucuronic acid) (Pierce *et al.*, 1992). Recently, a wall glycoprotein rich in ara and gal has been shown to be induced in response to stress in French bean (Millar *et al.*, 1992). This stress-inducible protein shows glycosylation at its N-terminus which is rich in both ara and gal (Millar *et al.*, 1992).

The trend in the galA levels observed in both resistant and susceptible interactions may be an indication of the disease progression and resistance in both susceptible and resistant cotton, respectively, after infection with *Xcm*. The observation that the galA

levels in the susceptible cotton cotyledons remained relatively high from 8 h post-inoculation to 24 h post-inoculation might indicate breakdown of galA-containing regions of the cotton cell wall (*i.e.*, pectic polysaccharides). This might have resulted from the activity of pectic enzymes. As was described in the review of literature, pectic enzymes have been associated with pathogenesis in plants. Pierce (unpublished observation) has measured PG activity in IWF prepared from both susceptible and resistant cotton at 24 h post-inoculation with *Xcm* and found PG activity from the susceptible but not the resistant line. So if pectic enzymes are active in the susceptible interactions then fragments that are rich in galA resulting from the action of these enzymes on their substrates should be generated.

In the case of *Xcm*-infected resistant OK 1.2 cotyledons, the galA content of the IWF was maximal at 8 h but gradually decreased shortly thereafter. At 16 h, 20 h, and 24 h post-inoculation the galA levels were not significantly different from that found at 0.5 h post-inoculation. The gradual decrease in galA levels might indicate that pectic enzymes responsible for generating fragments rich in galA may have been slowly inactivated. Pierce (unpublished observation) has investigated this and found that dialyzed IWF from *Xcm*-inoculated and mock-inoculated resistant or susceptible cotton cotyledons greatly reduced the PG from *Aspergillus japonicus* indicating the presence of a high mol wt pectic enzyme inhibitor. So the pectic enzyme(s) which may have generated the fragments rich in galA at 8 h post-inoculation may have been slowly inactivated by the same high mol wt pectic enzyme inhibitor which inactivated the PG from *Aspergillus japonicus*. The PG in the IWF from compatible interactions is active inspite of the presence of the high mol wt inhibitor. A possible explanation is that different pectic enzymes may have been induced in the compatible vs. incompatible interactions. The enzyme induced in the compatible interactions may not be susceptible to the inactivation by the high mol wt inhibitor. There are perhaps other possible explanations.

Preparation of Oligogalacturonide Derivatives and Separation of Derivatives that Differ from Each Other by a Single Galacturonic Acid Residue

Relatively stable derivatives of oligogalacturonides can be made through condensation of the reducing end of the oligogalacturonides with a fluorophoric molecule, 2-aminopyridine, to form the glycosylamine derivatives as shown in Figure 10 (Her *et al.*, 1987, Maness *et al.*, 1989; Maness *et al.*, 1991).

The derivative formed in this labeling procedure is the dehydration product of an aromatic amine and the galacturonosyl hemiacetal group. During the derivatization of the oligogalacturonides, excess 2-aminopyridine was necessary for efficient labeling. However, the non-bound 2-aminopyridine absorbs and fluoresces at the same wavelengths as the adducts and therefore obscures sample components. When such derivatization mixtures were injected directly into an HPLC apparatus equipped with a fluorescence detector, the interference from the 2-aminopyridine reagent front prevented detection of any labeled oligosaccharides. To circumvent this problem, the derivatization mixture was diluted to 20 times its original volume with 0.44 *M* acetic acid and applied to a cation-exchange column (Extract-Clean columns from Alltech) in the ammonium form and rinsed through with 400 μL water. The excess 2-aminopyridine was bound but not the labeled oligogalacturonides (Maness *et al.*, 1991). The retention of the excess 2-aminopyridine can be explained by its interactions with the Extract-Clean column matrix through hydrophobic effects as well as by ionic interactions with the sulfonate ion-exchange groups (Maness *et al.*, 1991). The hydrophobic interactions may be weakened by the hydrophilic nature and negative charge of the oligogalacturonides thus allowing the adducts to pass through without being retained.

To determine if the reaction involving 2-aminopyridine and sample clean-up with the Extract-Clean column was useful for minute quantities of oligogalacturonides, the

method was applied to the preparation of 100 pmol of the tetragalacturonide and pentagalacturonide. Glycosylamine derivatives of the tetragalacturonide and the pentagalacturonide were prepared using 10-μL reaction mixtures. After derivatization, the samples were diluted and passed through a 100-mg Extract-Clean column. The first 600 μL of effluent was collected and kept in an ice-bath until used. Aliquots of 100 μL were injected onto a TSK DEAE 2SW column, eluted as described in the experimental section, and their fluorescence intensity was monitored at 350 nm. The unreacted 2-aminopyridine peak, which was not retained by the TSK DEAE 2SW column, was on scale at the same sensitivity setting needed to see *ca.* 16 pmol of either the tetragalacturonide or the pentagalacturonide, both of which gave peaks of about one quarter of full scale. The signal-to-noise ratio for both oligogalacturonides was about 25:1.

The glycosylamine derivative of the hexagalacturonide (4.84 μg) was prepared in order to determine how much water was required to completely elute the derivatized oligogalacturonide from the Extract-Clean column. At the end of the incubation period, the reaction mixture was diluted and applied to the Extract-Clean column. The eluate was collected and immediately stored on ice. The micro-reaction vial was rinsed with 200 μL of distilled water, and the rinse was added to the same Extract-Clean column. The second eluate was collected separately and stored on ice. Another 200 μL of water was added to the Extract-Clean column, and the third eluate was collected separately and stored immediately on ice. Aliquots of the three eluates were injected on the PA-1 column and the peak corresponding to the hexagalacturonide was quantitated to determine which of the eluates contained the majority of the glycosylamine derivative of the hexagalacturonide. The first eluate collected contained 34% of the derivatized hexagalacturonide, the second eluate contained 64% of the derivatized oligogalacturonide, and the third eluate contained only 2% of the derivatized hexagalacturonide. In the subsequent analyses, the 100-mg Extract-Clean columns were eluted with three 200-μL portions of water following sample application in ~200 μL such that the total volume collected was ~800 μL. From hereon,

subsequent discussion regarding glycosylamine derivatives will mean that derivatized oligogalacturonides were prepared in 800 μL prior to quantitation by anion-exchange chromatography.

The glycosylamine derivative of the pentagalacturonide at pH 4.5 was stable for up to a week when stored at 4°C. When the pH of the solution containing the glycosylamine derivative of the pentagalacturonide was adjusted from pH 4.5 to a higher pH of 6.0 or 8.0 and stored at 4°C, the derivative was stable for up to four weeks. Four weeks was the longest time the stability of derivatives was tested. Glycosylamine derivatives left at room temperature over a 24 h period at pH 4.5 and pH 6.0 lost close to 50% of their labeled 2-aminopyridine while 35% of the label was lost under the same condition at pH 8. This observation suggested that the derivatized molecules should be kept cold to prevent decomposition of the derivatized oligogalacturonides. The pH of the oligogalacturonide derivatives was not adjusted from the original pH of the solution (*i.e.*, pH 4.5) since the derivatives were stored at 4°C and since the samples were analyzed within a few hours of being made.

In general, separation of each derivatized oligomer was achieved with the use of PA-1 anion-exchange chromatography as described in the experimental section. During the anion-exchange chromatography step, oligomers that differed from each other by a single galA residue could be differentiated. Using the potassium oxalate buffer (pH 7.80), the oligogalacturonides with dp = 6 to dp = 25 could easily be fractionated. With the sodium acetate gradient, oligogalacturonides with dp = 6 to dp = 16, could easily be eluted and distinguished from each other. Neutral molecules that had chromatographed with the oligogalacturonides during the previous purification procedures eluted early and did not interfere with the chromatography of the molecules of interest.

Molecules that Inhibited the Formation of Glycosylamine Derivatives

Experimental results showed that both CDTA and ammonium acetate inhibited the formation the glycosylamine derivatives. The condensation reaction was partially prevented by the presence of CDTA in the micro-reaction vial prior to the addition of the derivatizing agent, 2-aminopyridine. In one experiment, fraction 375 (45 μg) was mixed with CDTA, dried, and derivatized with 2-aminopyridine. The peaks of individual oligogalacturonides were compared with those from the same amount of fraction 375 that was mixed with water, dried, and derivatized with 2-aminopyridine. Derivatizing conditions and work-up were the same as described in the experimental section. When the peaks from the oligogalacturonides were summed up and compared to the sum of the peaks of the oligogalacturonides that were mixed with water only, the CDTA was found to have inhibited the condensation reaction by as much as 60%. The chemical structure of the CDTA does not indicate that it would compete with the oligogalacturonides for the 2-aminopyridine molecule so other factors are perhaps responsible. One such factor is the inability of CDTA to evaporate. CDTA, in the presence of water and oligogalacturonides, when subjected to repeated drying exhibited a gel-like appearance. This gel-like mixture may have resulted from CDTA-water interactions . Imbedded in this gel-like mixture were the oligogalacturonides which may have also interacted with the CDTA through hydrogen bonding. When the 2-aminopyridine reagent was added and the reaction was allowed to proceed, the gel-like material did not dissolve even after repeated vortexing. Mixing the solution inside the micro-reaction vessel helped break up the material, but the majority still did not dissolve even after prolonged incubation at 65°C. A possible reason for the high inhibitory effect of the CDTA on the condensation reaction is that it prevented the physical contact of the oligogalacturonides and the 2-aminopyridine since the material, once formed,

did not dissolve easily in the derivatizing agent. As will be discussed later, the problem with CDTA was solved by taking advantage of the behavior of the oligogalacturonides during a gel permeation chromatography step.

Ammonium acetate also interferes with the condensation reaction between 2-aminopyridine and oligogalacturonides. In one experiment, the undecagalacturonide (6.50 μg) was mixed with reagent grade ammonium acetate (200 m*M*, pH 5.2, 0.8 mL). The mixture was dried in order to remove most of the ammonium acetate, leaving about 10-15 μL. When the peak area of the derivatized undecagalacturonide was compared to that from undecagalacturonide that was mixed with water only prior to derivatization, the 10-15 μL of residual ammonium acetate was found to have caused a 90% reduction in the peak area of the undecagalacturonide. In another experiment, the dodecagalacturonide (25 μg) was mixed with reagent grade ammonium acetate (200 m*M*, pH 5.2, 0.6 mL) and dried for 15 h before being derivatized with the 2-aminopyridine reagent. The evaporation was judged to be complete when the bottom of the micro-reaction vial looked dry. As before, the reaction between the 2-aminopyridine and the dodecagalacturonide was carried out along with a control that had only the dodecagalacturonide and water. Comparisons between the sizes of the peaks observed showed that the ammonium acetate had still inhibited the reaction between the oligogalacturonide and the 2-aminopyridine by as much as 17%. A possible reason for the observed results is that the ammonium acetate, even after prolonged drying for 15 h, did not completely evaporate and competed with 2-aminopyridine for the oligogalacturonides. It is possible that an impurity in the reagent-grade ammonium acetate may have prevented its complete evaporation. The ammonia-oligogalacturonide adduct does not contain a fluorophoric group and is therefore not detected by the fluorescence detector. Use of high-purity ammonium acetate instead of a reagent-grade ammonium acetate seemed to help alleviate the problem. In one experiment, undecagalacturonide was mixed with high-purity ammonium acetate prior to derivatization. When the ammonium acetate was evaporated and the glycosylamine derivative was prepared, no inhibition from

the high-purity ammonium acetate was observed. The highly purified ammonium acetate did not evaporate right away. The problem of ammonium acetate interference in the 2-aminopyridine reaction was solved by the addition of a dialysis step prior to derivatization. The dialysis step removed the residual ammonium acetate while retaining the oligogalacturonides inside the dialysis membrane. Mort and co-workers (1991) found that when oligogalacturonides are dialyzed against water for as much as 24 h using a 1000 mol wt cut-off membrane, oligomers with degrees of polymerization of 3 and higher are essentially retained inside the dialysis bag.

Inhibition of Further Pectic Enzyme Activity Immediately after the IWF Are Collected

Immediately after collection of IWF, it was necessary to stop further reactions so that levels of oligogalacturonides found would reflect the actual values prior to IWF collection. At the same time, the method adopted to inhibit further enzymatic activities should not cause degradation of the oligogalacturonides and the inhibitor should be separable from the oligogalacturonides later during the purification process. Three different methods were tested. Boiling at 100°C for ~10 min is used to stop most enzyme-catalyzed reactions, and has been used to inactivate the pectic enzymes of *Erwinia carotovora* and *Chaetomium globosum* (Davis *et al.*, 1984; Amin *et al.*, 1986). However, boiling at 100°C caused decomposition of oligogalacturonides. In one experiment, the dodecagalacturonide was heated over boiling water for 10 min before preparing its glycosylamine derivative. When the peak areas of the dodecagalacturonides were compared, the dodecagalacturonide heated at 100°C for 10 min was found to have only 83% of the peak area of the control, indicating that heating for 10 min at 100°C was not a good choice for inhibiting further enzymatic activities.

Addition of trichloroacetic acid (TCA) to a final concentration of 2.5% (w/v) is another way to prevent enzymatic activities. TCA, when added to a final concentration of

2.5% (w/v) to a solution of dodecagalacturonide, did not cause degradation of the dodecagalacturonide as judged from the 2-aminopyridine reaction. However, during chromatographic purification on Toyopearl HW40S gel, the TCA exhibited an abnormal behavior. Two peaks were observed when TCA was injected on a HW40S semi-preparative column; the first peak was observed at ~65 min while the second peak was observed around 123 min. These two peaks exhibited retention times longer than water (52 min). Although TCA did not cause degradation of the oligogalacturonides, its usefulness is diminished because of its tendency to be retained in HW40S for a long time.

Sodium dodecyl sulfate (SDS), when added to the dodecagalacturonide at a final concentration of 0.25% (w/v), did not cause degradation of the oligogalacturonide as judged by the formation of the glycosylamine derivative compared to the control. SDS seemed to be the choice for inhibitng further pectic enzyme activities since its chromatographic behavior on HW40S allowed it to be separated from the oligogalacturonide. The ability of SDS to inhibit the PG from *Erwinia carotovora* was tested: SDS was added to the dodecagalacturonide solution to a final concentration of 0.25% (w/v) before the addition of 0.1 unit of PG activity. The reaction mixture was incubated for 30 minutes. The control samples were the solution of dodecagalacturonide and the dodecagalacturonide solution plus 0.1 unit of PG activity in the absence of SDS and incubated at the same time. When the control dodecagalcturonide plus PG was derivatized with 2-aminopyridine, no peaks were observed after PA-1 anion-exchange chromatography indicating that PG had digested the oligomer. The sample that contained dodecagalacturonide plus SDS and PG, when derivatized, had the same integrated area as the other control (*i.e.*, dodecagalacturonide only) indicating that the SDS prevented the breakdown of the oligomer by the PG *Erwinia carotovora.* SDS apparently disrupts the secondary, tertiary, and quaternary structure of proteins producing polypeptide in a random coil (Boyer, 1986). The detergent binds to the hydrophobic regions of the denatured protein chain in a constant ratio of about 1.4 g per g of protein or about one SDS molecule

for every two amino acid residues (Boyer, 1986; Voet and Voet, 1990). The SDS was separated from the oligogalacturonides in one of the chromatographic steps that will be discussed later on.

Levels of Galacturonic Acid in Untreated, Water-treated, and Mock-inoculated Cotton Cotyledons

A prerequisite to finding out whether oligogalacturonides are present during cotton-*Xcm* interactions was to determine whether galA-containing molecules can be extracted (eluted) efficiently from the cotton cotyledon intercellular spaces. Experiments were designed with the intention of finding out how much exogenous oligogalacturonides can be extracted back. However, it necessitated low levels of background galA-containing molecules in untreated cotyledons. It appeared that this was the case based on the sugar composition analysis at various times (Table 4) using water as extractant. However, subsequent data showed it was not the case. For example, oligogalacturonides were added at 1-2 nmol cm^{-2} in one of the experiments conducted. The background levels found in the control samples, eluted with one of the extractants described below, were 12-14 nmol cm^{-2} that accurate determination of the recovery was not possible. It was therefore necessary to devised a purification scheme (discussed later).

Table 5 is a summary of the levels of galA observed in mock-inoculated, water-treated, and untreated cotton cotyledons observed from various experiments done during the course of the investigation. GalA levels were from gas-liquid chromatographic analysis (GLC) and were from IWF collected using different extractants. The extractants used were water, imidazole (500 m*M*, pH 7.0), and *trans*-1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetraacetic acid hydrate (CDTA) (50 m*M*, pH 6.5). The chemical structures of imidazole and CDTA are shown in Figure 11.

CDTA is presumed to solubilize pectins by breakage of the calcium cross-bridges between adjacent pectate sequences within the pectins (Mort *et al.*, 1991). Komalavilas

and Mort (1989) found that chelating agents are effective in solubilizing pectin from cotton suspension culture cell walls. Imidazole is proposed to solubilize pectins by the same mechanism as the chelating agents (Mort *et al.*, 1991). It was shown that the non-protonated imino nitrogen of the imidazole ring of histamine can coordinate to Ca^{+2} strongly enough to form complexes that can be crystallized (Cole and Holt, 1986). Mort and co-workers (1991) proposed that even though the imidazole is mono-dentate, in high enough concentration (*i.e.*, 500 m*M*) it could compete with the carboxyl groups of the D-galacturonic acid in Ca^{+2} complex formation and thus disrupt the "egg box" structures within pectin proposed by Morris and co-workers (1982).

The values presented in Table 5 were either from 14- or 15-day-old untreated cotyledons or cotyledons treated with either water or water saturated with calcium carbonate (0.14 mg L^{-1}). Some were the controls from the sugar composition experiments with IWF collected from both compatible and incompatible interactions at various times after inoculation with *Xcm*. Others were control samples from a variety of experiments designed to quantitate the efficiency of various extractants in solubilizing exogenously-added oligogalacturonides from the cotyledon intercellular spaces. As mentioned earlier, those experiments were not quite conclusive since the recovery was based on the results of the glc analysis on the crude extracts that have background levels that were relatively high. The presence of the exogenously-added oligogalacturonides in the IWF extracts was not demonstrated since no purification scheme for the oligogalacturonides was yet established at that time. Finally, others were from preliminary experiments wherein the IWF were collected at 8 h post-infiltration of the cotyledons and the galA content of the crude mock-inoculated IWF were analyzed by glc. There were other drawbacks for analyzing galA levels directly by glc. Imidazole interfered with methanolysis, while CDTA was strongly retained by the column during chromatography and could build up in the column if repeated injections were made. IWF extracts using imidazole were dialyzed before methanolysis. CDTA did not dialyze away even after a prolonged dialysis (Mort *et al.*,

1991). Their results were confirmed in the following experiment. CDTA (50 μmol) was placed in a 1000 mol wt cut-off dialysis membrane and dialyzed against 4 L of water for 48 h. The water was changed three times over the 48 h period. Gas chromatography of the sample that was retained inside the dialysis membrane indicated that the CDTA had not dialyzed away over the 48 h dialysis period. Comparison of the effectiveness of each extractant could be more accurately assessed if each extract were subjected to a purification step.

A number of observations can be inferred from Table 5. It appears that CDTA is able to extract much more galacturonic acid-containing molecules than either water or imidazole. The lowest value observed from the CDTA was 12 nmol cm^{-2} while the highest value observed from either water or imidazole was 0.96 nmol cm^{-2}, which was IWF collected from untreated Ac 44E extracted with water. Disruption of the calcium-oligogalacturonide crosslink must be an important factor during oligogalacturonide solubilization since more is extracted with CDTA than with water or imidazole. Levels of galacturonic acid obtained by gas chromatography from both the water and CDTA extracts include both the polymeric molecules such as pectin and the galA monomer since the samples were not dialyzed. Values obtained from imidazole are underestimates since it did not include a portion of the monomeric form of galA. During dialysis of the monomeric galacturonic acid, ~40% is lost during a 24 h period when using a 1000 mol wt cut-off dialysis membrane (Mort *et al.*, 1991). Both mock-inoculated Ac 44E and OK 1.2 showed higher galA levels when IWF were prepared 24 h after mock-inoculation. The reason for the increase is not clear.

Can Oligogalacturonides Be Extracted Well from Cotton Cotyledons?

The data from the preceding section appear to show that CDTA is more efficient than either water of imidazole in extracting galA-containing molecules from the cotton

cotyledon intercellular spaces. A prerequisite to finding out whether oligogalacturonides are present during cotton-*Xcm* interactions was to determine whether exogenous oligogalacturonides could be extracted (eluted) efficiently from the cotton cotyledon intercellular spaces. To address this question, cotyledons were infiltrated with a known amount of one of the phytoalexin-elicitor-active oligogalacturonides (*i.e.*, undecagalacturonide, dp = 11).

The undecagalacturonide-rich solution (0.5 mg mL^{-1}, pH 7.0) was applied, close to the middle of the photoperiod, to the cotyledons of 2-week-old highly resistant cotton cultivar WbM (0.0) filling the intercellular spaces. The calculated amount of undecagalacturonide-rich fraction added was 4.5 μg cm^{-2} (2.3 nmol cm^{-2}) plant tissue. This amount of the was calculated from the value of the intercellular volume. This value was ~9 μL cm^{-2} and was obtained by computing (initial cfu cm^{-2}) / (inoculated cfu mL^{-1}) from experiments in which sample discs were harvested within a few minutes after inoculation of cotton cotyledons with *Xcm* (see Table 6). The value ~9 μL cm^{-2} was chosen to avoid error due to any bacterial multiplication between infiltration and sampling. The concentration of 0.50 mg mL^{-1} undecagalacturonide-rich fraction was used because it was the concentration of the dodecagalacturonide that reproducibly elicited accumulation of stress metabolites. The oligogalacturonide was extracted into IWF immediately after the water of infiltration had transpired. The high background levels of galA in extracts (Table 5) compared to the amount added (2.3 nmol cm^{-2}) necessitated a purification scheme. The oligogalacturonides were purified by selecting against both low mol wt and high mol wt molecules through two chromatographic steps.

The same solvents from the preceeding section were tried as extractants. They were water, imidazole (500 m*M*, pH 7.0), and CDTA (50 m*M*, pH 6.5). A second extraction of the same oligogalacturonide-treated cotyledons was done to check the efficiency of the first extraction.

Amounts of IWF representing 15 cm^2 were processed for each extract (*i.e.*, CDTA,

imidazole, and water) and injected on the semi-preparative HW40S column. Control samples that contained 67.5 μg undecagalacturonide in water were also subjected to the same chromatography on HW40S to evaluate the efficiency of each extractant. A second set of control samples from cotyledons infiltrated with water only was also subjected to the same fractionation procedure.

Separation of Oligogalacturonides from CDTA and Imidazole on Toyopearl HW40S

An initial gel permeation chromatography on Toyopearl HW40S was employed to remove components interfering with oligogalacturonide quantitation. In the HW40S gel permeation chromatography step, the oligogalacturonides (*e.g.*, ~2000-2400 g mol^{-1}; dp = 10-13) behaved like molecules so much larger than their actual molecular weight that it was easily possible to separate them from components of the extracting medium. Evidently, oligogalacturonides, which are rod-like rather than random coils, behave as though they are much larger than globular molecules with corresponding molecular weights (*e.g.*, pullulans and dextrans) because of the electrostatic repulsion between the adjacent D-galA residues and restricted rotation around the α-1,4-glycosidic linkages (Fishman *et al.*, 1984). Fishman and co-workers (1984) found that even at ionic strengths of 0.1 and above, pectins behave as segmented rods. This anomalous chromatographic behavior of pectic substances was ascribed also to ionic exclusion; that is, exclusion of the negatively charged pectic molecules from the pores of the gel by the small number of fixed negative charges on the gel. Thibault (1980) reported that the elution position of D-galA on BioGel P-2 approached the void volume as the ionic strength of the medium approached 0. As described by Mort and co-workers (1991), D-galA-containing oligomers behave upon size-exclusion chromatography as much larger molecules than one would expect, unless aggregates are disrupted before the chromatography and the ionic strength of the elution buffer is at least 0.1.

Two groups of positive peaks were observed using a refractive index detector when crude CDTA-, imidazole-, and water-extracts were injected on the Toyopearl HW40S column. These groups of peaks were seen only in samples extracted from the cotyledons. The control representing the amount of undecagalacturonide standard contained in 15 cm^2 IWF showed only the one positive peak corresponding to the undecagalacturonide. All injections showed one negative peak due to water. The positive peaks observed from the CDTA-extracts were taller relative to those of the imidazole- and water-extracts. The first group of positive peaks included compounds that were eluted close to the void volume, V_o, (see Figure 12, and Figure 13 A, C, and E) while the second group included ones that were eluted near and with the included volume, V_i (see Figure 12, and Figure 13 B, and D). The oligogalacturonides were eluted close to the V_o (Figure 12). During HW40S chromatography in 200 m*M* ammonium acetate, pH 5.2, the extractants (*i.e.*, imidazole, CDTA, and water) along with the other smaller molecules, such as glucose and sucrose, chromatographed with or near the V_i. The concentration of 200 m*M* ammonium acetate was chosen because it prevents aggregation of the oligogalacturonides (Mort *et al.*, 1991). The evidence that oligogalacturonides chromatograph close to the V_o was provided by injecting purified oligogalacturonides on the HW40S column.

Typical retention times of some molecules of interest on a semi-preparative HW40S column (200 m*M* ammonium acetate, pH 5.2 at 0.5 mL min^{-1}) were as follows (also see Figure 14): oligogalacturonide fraction 375 (a mixture of oligogalacturonides dp = 8-16), 28 min; dodecagalacturonide, 28 min; hexagalacturonide, 33 min; tetragalacturonide, 36 min; SDS, 47 min; CDTA, 43 min; and imidazole, 47 min. Typical retention times of some molecules on a preparative HW40S column (200 m*M* ammonium acetate, pH 5.2 at 2.0 mL min^{-1}) were as follows (also see Figure 14): citrus pectin from Sigma Chemicals, 24 min; fraction 375, 26 min; heptagalacturonide product of PL, 30 min; pentagalacturonide product of PL, 30 min; CDTA, 41 min; and monomeric galA, 43 min.

The majority of compounds rinsed out from the cotton cotyledon intercellular

spaces by all three extractants were found in the second group of peaks (Figure 12). The first group of peaks, which included the oligogalacturonides, was collected, dried, and purified further. This fraction containing the oligogalacturonides included the high mol wt molecules.

In summary, oligogalacturonides can be separated from solutes of the extracting medium such as CDTA and imidazole through chromatography on Toyopearl HW40S.

Separation of Oligogalacturonides from High-molecular Weight Molecules

Gel permeation chromatography on TSK2000SW allowed further purification of the oligogalacturonides. The fraction collected from the HW40S was redissolved in 1 mL sterile water and filtered as described in the experimental section. During chromatography on TSK2000SW column, high mol wt molecules, such as citrus pectin, chromatographed with the V_o. The oligogalacturonides, however, chromatographed near the V_i (see Figure 15). Typical retention times of compounds on TSK2000SW were as follows (also see Figure 16): citrus pectin from Sigma Chemicals (mol wt 23,000-71,000), 13 min; arabinogalactan, 16 min; bovine serum albumin, 17 min; nanodecagalacturonide, 17 min; oligogalacturonide fraction 375, 18 min; dodecagalacturonide, 19 min; heptagalacturonide product of PL, 19 min; CDTA 21 min; and glucose, 22 min.

The fractions co-chromatographing with the undecagalacturonide-rich fraction (14-24 min) were collected, pooled, dried, and redissolved in 1 mL sterile water (Figure 15). An aliquot of the purified oligogalacturonides representing IWF from 6-10 cm^2 of cotyledon from the TSK2000SW step was withdrawn and the amount of recovered undecagalacturonide-rich fraction was quantitated by the 2-aminopyridine reaction and subsequent chromatography on a PA-1 column. The efficiency of each extractant was calculated by the following: the corrected peak areas of the undecagalacturonide and decagalacturonide were obtained by subtracting the peak areas representing the

undecagalacturonide and decagalacturonide from PA-1 chromatography of the control sample infiltrated with water from the peak areas in chromatograms from oligogalacturonide-treated samples, both peaks normalized to 15 cm^2 of cotyledon. The efficiency was computed as the ratio of the corrected peak area from IWF samples to the peak area determined for 67.5 μg of undecagalacturonide that had been subjected to the same chromatographic steps as the IWF extracts. This formula was used for all extracts. No undecagalacturonide and decagalacturonide were found in the control cotyledons infiltrated with water and extracted with various solvents. In later experiments, oligogalacturonides were found. The amount of the undecagalacturonide-rich fraction added per cm^2 was so much greater than the amount of undecagalacturonide per cm^2 in the control samples that at the detector settings used it would be difficult to detect the oligogalacturonides in the control cotyledons. The results are tabulated in Table 7. CDTA was the most efficient of the three extractants examined. Close to 86% of extractable undecagalacturonide was extracted during the first extraction step. The second CDTA extraction amounted to an additional 10% of the undecagalacturonide originally added to the cotyledons.

As shown in Table 7, water and imidazole were, respectively, 1/4 and 1/9 as efficient as CDTA in extracting the undecagalacturonide-rich fraction. A second extraction with water did not appreciably increase the amount of recovered undecagalacturonide-rich fraction. In contrast, a three-fold increase in recovery was observed when the undecagalacturonide-treated tissue was re-extracted with imidazole.

The recovery of the dodecagalacturonide from both the HW40S and TSK2000SW columns was also investigated. In an experiment, 23 nmol of the dodecagalacturonide was injected twice on both the HW40S and TSK2000SW columns. The dodecagalacturonide eluting from the two gel permeation columns was collected and diluted to 8 mL with 200 m*M* ammonium acetate, buffered at pH 5.2. Two control samples were made which contained 23 nmol each of the dodecagalacturonide in 8 mL of the same buffer. From each

sample, 1-mL aliquot was dried in a 0.8-mL micro-reaction vessel and derivatized with 2-aminopyridine. The percent recovery from each gel permeation column was computed from the PA-1 chromatography of the glycosylamine derivative of the dodecagalacturonides as follows: the peak area corresponding to the dodecagalacturonide from each of the fractions collected was divided by the peak area from the control samples and multiplied by 100. The percent recovery from the HW40S column was from 60-70%. The percent recovery from the TSK2000SW column was from 46-50%. The expected recovery from the HW40S to the TSK2000SW step was ~30%. Including the extraction with CDTA, the expected overall recovery was ~22%. A table of estimated recovery of oligogalacturonides extracted with CDTA from cotyledons and purified by gel permeation chromatography is presented in Table 8. Overall recovery for the CDTA extracts, which includes the gel permeation chromatographies on both the HW40S and TSK2000SW columns and all of the other manipulations involved to the point of derivatization with 2-aminopyridine was close to 6%. The overall recovery was calculated by taking the ratio of the peak areas of the glycosylamine derivatives of the undecagalacturonide and decagalacturonide recovered with CDTA from 15 cm^2 of cotyledon and subjected to the chromatographic steps mentioned earlier compared to the peak area from the glycosylamine derivative of 67.5 μg undecagalacturonide-rich fraction that was not subjected to the gel permeation chromatographic steps (HW40S and TSK2000 SW) mentioned above.

However, the overall recovery (*i.e.*, 6%) obtained from the recovery studies with the undecagalacturonide-rich fraction was only 1/5 of the expected recovery. A possible explanation for the less than expected overall recovery of the undecagalacturonide-rich fraction could be the number of physical transfers from one container to another container which may have resulted in the non-specific adsorption of the oligogalacturonides on the surface of the container. In the subsequent studies with the IWF, the containers used for collecting fractions were treated with Sigmacote silanizing solution from Sigma Chemicals to minimize non-specific loss of oligogalacturonides.

Results of the experiments performed above are different from those found by Mort and co-workers (1991) when imidazole (500 m*M*, pH 7.0) and CDTA (50 m*M*, pH 6.50) extracted the same amount of galA-containing molecules from cotton suspension cell walls. A possible explanation is the difference in the length of time given for extraction. Mort and co-workers (1991) extracted the pectic fragments for at least 15 h. In contrast, in the experiments with undecagalacturonide-rich fraction mentioned above, the IWF were prepared immediately after the undecagalacturonide-treated cotyledons were infiltrated under vacuum with the various extractants. The extractants did not remain within the cotyledons' intercellular spaces for more than an hour. Maybe not enough time was allowed for imidazole to interact with the galA-containing molecules. Another possibility is that the relative affinities or strength of binding for Ca^{+2} is apparently different: it is likely to be stronger between CDTA and Ca^{+2} than it is between Ca^{+2} and imidazole. Although the molar concentration of imidazole used for extraction was 10x that CDTA (*i.e*, 500 m*M* vs 50 m*M)*, the magnitude of the K_d (dissociation constant) for Ca^{+2}/Imidazole is most likely larger than that of Ca^{+2}/CDTA that it could upset the difference in the molar concentrations of the extractants used.

Results of the recovery experiments indicate that CDTA (50 m*M*, pH 6.50) is the solvent of choice for maximal extraction of non-covalently-bound oligogalacturonides from the cotton cotyledon intercellular spaces. CDTA was able to extract ~70% of undecagalacturonide-rich fraction that was added to the cotyledon.

Preliminary Analysis for the Presence of Oligogalacturonides in IWF Prepared at Various Times

Initially, IWF were prepared at 8 h post-inoculation from cotton cotyledons of WbM (0.0) (resistant) inoculated with *Xcm* (9.00×10^7 cfu mL^{-1}). The IWF were prepared as described in the experimental section and were subjected to the fractionation procedures mentioned earlier (*i.e.*, chromatography on HW40S and TSK2000SW gel

permeation columns). CDTA was the extractant used. The CDTA, along with other smaller molecules, was removed during the first fractionation step. High mol wt molecules were removed during the second gel permeation chromatography. The purified fraction collected after the TSK2000SW fractionation step was derivatized with 2-aminopyridine directly without dialyzing, and PA-1 anion-exchange chromatography of the derivatives was run in a sodium acetate gradient as described in the experimental section. Figures 17 and 18 show that oligogalacturonides were present at 8 h when cotyledons were infiltrated with *Xcm,* including oligogalacturonides that are of phytoalexin-elicitor-active sizes. IWF were also prepared from both *Xcm*-treated and mock-inoculated WbM (0.0) (resistant) and WbM (4.0) (susceptible) lines at 8 h post-inoculation and also from Im 216 (resistant) and Ac 44E (susceptible) lines at 24 h post-inoculation. In all experiments, the presence of oligogalacturonides from both *Xcm*-treated and mock-inoculated cotton cotyledons was demonstrated. In the above mentioned experiments, IWF were also prepared from the two untreated (uninoculated) cotyledons of the four cotton lines. Surprisingly, untreated cotyledons contained oligogalacturonides, including oligogalacturonides of sizes that are phytoalexin-elicitor-active! During these experiments, accurate oligogalacturonide levels could not be assessed at that point since no internal standard was added to the aliquots of IWF fractionated and the samples were not dialyzed following TSK2000SW chromatography to prevent interference from ammonium acetate.

The pectic nature of the molecules producing the peaks observed during the PA-1 anion-exchange chromatography was established by a number of observations. For example, purified IWF obtained from *Xcm*-treated cotyledons exhibited peaks during PA-1 anion-exchange chromatography that corresponded to those from oligogalacturonides obtained from autoclave-hydrolysis of polygalacturonic acid (Figures 17 and 18). Those peaks did not appear when the same amount of purified IWF from *Xcm*-treated cotyledons was incubated first with a pectic enzyme (*i.e.*, 0.10 unit of the PG from *Erwinia carotovora*) prior to derivatization with 2-aminopyridine (Figure 17). Controls were run

side by side with this experiment. They included undecagalacturonide solutions that were incubated with and without pectic enzyme. Just as with the purified IWF, the peak that corresponded to the standard undecagalacturonide disappeared when the undecagalacturonide was incubated with the pectic enzyme (chromatogram not shown). Furthermore, the retention times of the peaks observed matched the retention times observed for standard oligogalacturonides obtained from autoclave-hydrolysis of polygalacturonic acid on the PA-1 anion-exchange column used (Figure 18). This was observed whether the IWF was chromatographed in sodium acetate or potassium oxalate solution. Also, the manner in which the chromatography of the IWF was performed, the PA-1 anion-exchange column will retain only negatively-charged molecules and will allow neutral molecules to elute early. The long retention times observed for those peaks that disappeared after PG treatment indicated that the molecules were negatively charged. Finally, the pattern of the peaks observed was similar to that observed in the standard mixture derived from autoclave-hydrolysis of polygalacturonic acid. Early eluting peaks (*eg.*, dp = 6-11) observed from the purified IWF displayed a major and a minor peak for each oligogalacturonide which were similar to those observed from standards obtained by autoclave-hydrolysis of polygalacturonic acid. The major and minor peaks are believed to be the ß- and α- forms of the derivatized oligogalacturonides (Her *et al.*, 1987).

Quantitation of Oligogalacturonide Levels Observed in Uninoculated, Mock-inoculated, and *Xcm*-inoculated Resistant WbM (0.0) and Susceptible WbM (4.0) Cotton Cotyledons

IWF were collected at various times from both the susceptible WbM (4.0) and resistant WbM (0.0) cotyledons to find out if the two lines would exhibit different levels of oligogalacturonides when challenged with *Xcm*. Specifically, the levels of elicitor-active sizes of oligogalacturonides in both the resistant and susceptible plants were compared. CDTA was the extracting solvent used for these experiments. IWF were collected at 8 h

post-inoculation because the galA levels found during the time course experiments analyzing IWF sugar composition, from both susceptible and resistant cotton lines, were maximal at this time. IWF were also collected at 17 h post-inoculation because Venere and co-workers (1984) reported that pectic enzyme activities in extracts derived from *Xcm*-infiltrated susceptible cotton lines were close to maximal at that time. IWF were also collected at 4 h post-inoculation because pectic enzyme activities in extracts derived from *Xcm*-inoculated resistant cotton lines were maximal at this time (Venere *et al.*, 1984). As described in the experimental section section, SDS was added to each IWF collection tube before CDTA-infiltrated plant materials were centrifuged. This step was done in order to insure that no pectic enzyme activity occurred once the IWF were collected.

In order to accurately assess the levels of oligogalacturonides, an internal standard was added. The internal standard chosen was added immediately after a known aliquot of the IWF, equivalent to a known surface area, was withdrawn for subsequent analysis. The oligogalacturonide with a dp = 6 derived from treating polygalacturonic acid with PL was chosen because it satisfied several requirements of an appropriate internal standard. First, based on previous observations, the internal standard chosen was not a normal constituent of any purified IWF derived from *Xcm*-inoculated, mock-inoculated, or untreated cotyledons. No peak was observed in PA-1 chromatograms between the saturated heptagalacturonide and the saturated octagalacturonide. In the chromatographic conditions used, the standard 4,5-unsaturated hexagalacturonide chromatographed between the standard saturated heptagalacturonide and the saturated octagalacturonide. Second, with its retention time equal to ~30 min, PL product with dp = 6 chromatographed within the first group of peaks that was collected (*i.e.*, 22-36 min) during the first fractionation step of IWF on the HW40S column and was therefore included in the fraction that was collected and purified further. Third, during further purification on the TSK2000SW column, the dp = 6 PL product again chromatographed with the fraction of interest.

The 4,5-unsaturated PL product with dp = 7 was also screened as a possible

internal standard. It was not chosen because it chromatographed close to the saturated undecagalacturonide during PA-1 anion-exchange chromatography in potassium oxalate buffer and it partially interfered with the peak that corresponds to the saturated decagalacturonide. The 4,5-unsaturated PL product with dp = 5 was also tried as a possible internal standard. It also co-chromatographed with a saturated oligogalacturonide (*i.e.*, dp = 6) that was in previously observed in IWF. However, the PL-derived oligogalacturonide with dp = 5 did not chromatograph with or close to any of the oligogalacturonides of interest (*i.e.*, oligogalacturonides with dp = 10-13). The PL-derived oligogalacturonide with dp = 5 could be used as a suitable internal standard as long as a sufficient amount were used so that the peak due to the saturated oligogalacturonide with dp = 6 was negligible. One disadvantage of both the 4,5-unsaturated PL products with dp = 5 and dp = 7 was that a relatively large amount of both would be needed in order to ascertain that the peak area contributions due to the oligogalacturonides that they co-chromatograph with or partially interfered with would be negligible. However, the amount of internal standard added should not be too large since a large amount of either dp = 5 or dp = 7 relative to the other peaks present might not only affect the hexagalacturonide or the decagalacturonide but also the neighboring peaks. This was particularly true for the dp = 7 PL product since the retention times of the decagalacturonide and the undecagalacturonide were very close during chromatography in both solvent systems used. Another disadvantage of both the PL products with dp = 5 and dp = 7 is that a prior knowledge of hexagalacturonide and decagalacturonide contents of the IWF collected should be known in order to accurately assess how much PL products with dp = 5 or dp = 7 to add. The PL product with a dp = 6 does not suffer from this disadvantage as it does not directly interfere with any of the saturated oligogalacturonides.

IWF collected at 8 h and 17 h post-inoculation were from 14-day-old cotyledons planted at the same time. IWF were also collected from 14-day-old plants of the same set, from cotyledons that were mock-inoculated with calcium carbonate-saturated water

solution, *i.e.* the bacterial suspension medium. There were two replicates per treatment per line. IWF from a replicate (~220 cm^2) were collected from ~25 cotyledons from ~23 plants. *Xcm*-treated plants were infiltrated with an inoculum concentration of 1.02 x 10^8 cfu mL^{-1}. The IWF collected from untreated (uninoculated) cotton plants were from the same set of plants, but were collected when the plants were 13 days old. Average yield of IWF collected at 8 h and 17 h post-inoculation was ~18 μL cm^{-2}. Correction for dilution by rinses of the IWF collection tubes gave a value of ~15 μL cm^{-2}. During collection of IWF, there were numerous samples to be processed at one time and time was not taken to blot dry the samples completely, thus the yield appears greater than the estimated intercellular volume of 9 μL cm^{-2}. In order to stop further enzymatic activities that might occur during the time the extractant was in the cotyledon intercellular spaces, it was necessary to work as fast as possible.

Mock-inoculated cotyledons were infiltrated with the bacterial suspension medium 3.6 h into the photoperiod and IWF were prepared 8 h later. Also, mock-inoculated cotyledons were infiltrated with the bacterial suspension medium 9.5 h into the photoperiod and IWF were prepared 17 h later. *Xcm*-inoculated resistant cotyledons were inoculated at 6.5 h into the photoperiod and IWF were prepared 8 h later *Xcm*-inoculated cotyledons were inoculated at 8 h into the photoperiod and IWF were prepared 17 h later. IWF from untreated cotyledons were collected at 6.5 h into the photoperiod. The times of inoculation were calculated by taking the midpoint of the start of inoculation and the end of the inoculation, and time of IWF preparation was the start of the centrifugation after the cotyledons had been infiltrated with CDTA.

Another set of experiments was conducted in which IWF were collected at 4 h and 24 h post-inoculation, also from 14-day-old cotyledons, but using a different set of plants. For this set of experiments in general, a replicate represented IWF collected from 15 cotton plants (30 cotyledons). Average total surface area of each replicate was ~200 cm^2. The average yield of IWF was ~15 μL cm^{-2}. After correction for dilution due to rinsings, the

yield was ~12 μL cm^{-2}. Mock-inoculated cotyledons were infiltrated with the bacterial suspension medium 7.5 h into the photoperiod and IWF were prepared 4 h later. *Xcm*-inoculated cotyledons were inoculated 5.5 h into the photoperiod and IWF were collected 4 h later. *Xcm*-infiltrated cotyledons were infiltrated (inoculated) at 4.3 h into the photoperiod and IWF were collected 24 h later. IWF were also collected from untreated (uninoculated) 15-day-old cotyledons at 3.5 h into the photoperiod from the same set of plants that were used for collecting IWF at 4 h and 24 h post-inoculation.

Precautionary procedures were routinely followed in order to minimize possible microbial contaminations. These procedures included the following: 1) IWF were kept in a autoclave-sterilized container and stored routinely at -20°C. When needed, IWF were slowly thawed in an ice-filled bucket underneath a flame-lit bunsen burner in order to maintain a sterile environment, 2) both gel permeation columns were flushed routinely with 5% SDS (w/v) and then with 0.5% (w/v) sodium azide for at least 4 h prior to using the columns and again after the columns were used. Both columns were routinely stored in 0.5% (w/v) NaN_3. These precautions were found to be necessary. In one instance, a column was inadvertently left in the sodium acetate buffer prior to an experiment designed to purify the oligogalacturonide with dp = 19 on the semi-preparative HW40S column. Supposedly purified fractions eluting from the HW40S column were collected and derivatized with 2-aminopyridine reagent. Chromatography on PA-1 anion-exchange column showed numerous peaks which chromatographed ahead of the nonadecagalacturonide (dp = 19) suggesting that the these molecules were negatively-charged but had dp's less than the nonadecagalacturonide. The early eluting peaks were not present to the same extent in the original dp = 19 suggesting that pectic-enzyme producing microorganisms had grown inside the HW40S column, 3) when not in use, both the HPLC apparatus, including the injector port, and the refractive index detector were stored in 70% ethanol (v/v). The syringes used during chromatographic injections were left filled with 70% ethanol when not in use. The plungers of the syringes used were

immersed in 70% ethanol for a few minutes prior to chromatography. Fractions collected from the gel permeation columns were stored at -20°C.

A preparative HW40S column, rather than a semi-preparative column, was used for all IWF analyzed in the presence of an internal standard (*i.e.*, dp = 6). The internal standard (4.0 nmol) was added to volumes of IWF equivalent to either 50 or 80 cm^2 of plant material. The fraction eluting from the HW40S column from ~22 ± 1 min to 36 ± 1 min post-injection was collected (Figure 19). Based on previous injections of standards on HW40S, this fraction would include from high mol wt polymeric materials down to the relatively smaller oligogalacturonides such as the pentagalacturonide. The collected fraction at that point would have a volume of ~28 mL. The fraction was divided into five 8-mL containers before they could be dried. The fraction was not collected directly in the 8-mL containers since the internal standard has a retention time on the column slightly behind that of the molecules of interest. If this procedure had been followed, one or two containers would have contained the majority of oligogalacturonide of a certain size while another container would have contained the majority of the internal standard. This would be risky whenever spills occurred from one or several of the 8-mL containers since it would affect the calculated levels of oligogalacturonides in the IWF. Collecting the fraction into one container was safer in the event of spillage since equal amounts of both the oligogalacturonides and the internal standard would be lost thereby maintaining the ratio of a particular oligogalacturonide to the internal standard constant. The error therefore in the subsequent calculations was minimized. The HW40S fractions were dried overnight. These samples could not be injected directly for the next chromatography step since they needed to be pooled into one container and then dried again.

Injection, following concentration, of the 22-36 min fraction collected during the HW40S chromatography on the TSK2000SW gel permeation column allowed further purification of the oligogalacturonides (Figure 20). The chromatograms shown in Figure 21 were from an IWF that had retained a fraction of ammonium acetate prior to

fractionation (A) and an IWF that had been thoroughly dried prior to fractionation (B) in TSK2000SW. There was no observable difference in the overall recovery of these samples. Samples that have retained a fraction of ammonium acetate prior to chromatography on TSK2000SW were more numerous. The fraction eluting from the TSK2000SW column from 14-24 min included the oligogalacturonides while the polymeric or high mol wt pectic materials, such as molecules the size of citrus pectin, would have eluted between 8-14 min post-injection. The collected fraction was small enough in volume (*i.e.*, ~5 mL) that it was collected in one 8-mL vial.

Overall recoveries, determined by PA-1 anion-exchange chromatography, from the HW40S and TSK2000SW fractionations and all other manipulations involved to the point of derivatization with 2-aminopyridine ranged from 7% to 28% (n = 39). The average overall recovery was 15.1% ± 5.6 (% recovery ± standard deviation). Glassware treated with Sigmacote silanizing solution from Sigma apparently helped increase the overall recovery, since lower recoveries were generally obtained when glassware were not silanized as in the recovery studies with the undecagalacturonide, which had an overall recovery of ~6%.

As was expected from previous observations made with IWF collected from various cotton-*Xcm* interactions, oligogalacturonides of varying dp's were present at various times in all the interactions studied. A sample PA-1 chromatogram of IWF from *Xcm*-inoculated cotyledons is shown in Figure 21. The level of each oligogalacturonide found was calculated by the following: the ratio of the peak area of an oligogalacturonide to the peak area of the internal standard of the same chromatogram was computed (ratio 1). The ratio of the peak area of the undecagalacturonide to the peak area of the internal standard from a chromatogram of a mixture that contained only a known amount of standard undecagalacturonide plus the same amount of the internal standard that was added to the aliquots of IWF taken for analysis was computed. Ratio 1 was divided by ratio 2. The ratio obtained (ratio 1/ratio 2) was multiplied by the amount of the standard

undecagalacturonide that was mixed originally with the internal standard. The result is the amount of an oligogalacturonide present in the aliquot of IWF analyzed. Finally, the tissue level of oligogalacturonide was obtained by dividing the amount of oligogalacturonide by the surface area represented by the aliquot of IWF taken.

The presence of the oligogalacturonides from both the untreated (uninoculated) WbM (0.0) (resistant) and the untreated WbM (4.0) (susceptible) cotyledons was also demonstrated (Table 9). In preliminary experiments (without an internal standard), the presence of the oligogalacturonides in IWF was also demonstrated from both the untreated cotyledons of resistant Im 216 and those of susceptible Ac 44E had been observed (data not shown). The demonstration of the presence of the oligogalacturonides in the untreated cotyledons could be an indication of the dynamic nature of the plant cell wall. The cotyledons used for these experiments were 13-15 days old. The cotyledon at that age is still expanding. The increase in size of the cotyledon is particularly evident from about 11 days after planting to about 14 days after planting. Most of the experiments were started 13-14 days after planting. Sections of homogalacturonan that are precursors to plant cell wall or middle lamella are expected to be larger than elicitor-active oligogalacturonides (Mort, personal communication), but oligogalacturonides might have resulted from the turning-over of galA-containing components of the plant cell wall or middle lamella and have resulted from the possible scenario described below.

As discussed by Brett and Waldron (1990), the growth of plant cells depends on the interaction between turgor pressure, which presses the protoplast against the cell wall, and the mechanical strength of the wall, which tends to prevent any increase in volume. It is now known that cell expansion, which can be stimulated by plant growth regulators such as auxin, is governed to a large extent by selective, closely-controlled weakening of certain areas of the cell wall. However, the region(s) of the plant cell wall that is involved in such process is not known. Plant cell-wall extension can be explained through the acid growth hypothesis (Brett and Waldron, 1990) which predicts that due to the extrusion of hydrogen

ions across the plasma membrane, predicts acidification of the plant cell wall results in an increase in wall extensibility. This increase in wall extensibility may be due to the weakening of one or more types of bond within the wall. It is not clear what type of bonds, covalent or non-covalent, are weakened. However, there seems to be a general agreement that the bonds weakened are those located in the cell wall matrix, or the cellulose microfibril-matrix interface, but not within the cellulose microfibrils. Calcium bridges in the cell wall matrix are possible sites of weakening, since a decrease in pH in the physiological range causes displacement of calcium from the wall. However, it is not certain whether the calcium ions concerned are bound to polygalacturonic acid or to some other negatively-charged species, such as glycoprotein or glucuronoxylan (Brett and Waldron, 1990).

A scenario for the presence of the oligogalacturonides could be hypothesized. Since the cotyledon is growing rapidly at 11-14 days after planting, it is reasonable to assume that the cell wall is expanding at these times. It is possible to envision that the acidification has weakened the Ca^{+2}-homoglacturonan interactions (*i.e.*, disruption of the "egg-box" structures) thus exposing the homogalacturonan region, a matrix polysaccharide, to enzymic attack. Acidification of the cell wall medium to pH 5.0, predicted by the acid growth hypothesis, could trigger the activation of plant PGs, pectic enzymes that are particularly active at this pH (Brett and Waldron, 1990). The presence of PGs has been documented in a number of plants. PGs are especially active during fruit ripening, which results in degradation of the plant cell wall. However, there are no studies made that correlate pectic enzyme activity to leaf expansion. PG activity has been shown to rise during fruit softening in peaches, pears, avocados, and tomatoes (Brett and Waldron, 1990; Tucker and Grierson, 1982; DellaPenna *et al.*, 1986; Bird *et al.*, 1988). It would be interesting to find out whether there is a correlation between auxin production and induction of PG activity in expanding cotton cotyledons. It remains to be investigated whether there is a pectic enzyme activity expressed in untreated cotton cotyledons. From

the recovery studies with the undecagalacturonide-rich fraction, no significant degradation had occurred, indicating that there was no easily observable PG enzyme activity during the time the undecagalacaturonide-rich fraction was in the cotyledon intercellular spaces. Shorter oligogalacturonides (*i.e.*, shorter than the undecagalacturonide plus the decagalacturonide which is present in the undecagalacturonide-rich fraction) were not observed in the PA-1 anion-exchange chromatograms. During the recovery studies, however, the undecagalcturonide-rich fraction remained in the cotyledon intercellular spaces for only a short period of time (<30 min) before the intercellular spaces were infiltrated with various extractants, perhaps not enough time for the PG to act on the substrate if it was present and active at the same time. One way to find out if PG is present in cotton cotyledon intercellular spaces is to prepare IWF at various times after infiltration of cotyledons with an oligogalacturonide. The IWF collected at various times could be purified, derivatized, and analyzed by PA-1 anion-exchange chromatography as described earlier. The presence of a mixture of oligogalacturonides shorter than the original oligogalacturonide infiltrated into the cotyledons would indicate pectic enzyme activity. Pierce (personal communication) found that IWF obtained from *Xcm*-treated cotton cotyledons had to be incubated with the substrate overnight in order to measure significant pectic enzyme activities. Hopper and co-workers (1975) incubated the reaction mixtures from culture filtrates of *Xcm* for 24 h in order to measure pectic enzyme activity. The picomolar quantities of the oligogalacturonides observed might have resulted from low activity of a plant pectic enzyme. However, further examination of the chromatograms from the PA-1 anion-exchange chromatography during the recovery studies with the undecagalacturonide-rich fraction appears to indicate the presence of an exopolygalacturonase. This observation was prompted by the following result: the ratio of the peak area of the undecagalacturonide to the decagalacturonide had decreased in the undecagalacturonide-rich samples that had passed through cotyledons relative to the ratio observed in the control standards (*i.e.*, undecagalacturonide-rich fractions that had

undergone chromatographic purification but were not IWF samples). The ratio had decreased by ~60% when the peak area of the main anomer of the undecagalacturonide was divided by the peak area of the decagalacturonide. The presence of exopolygalacturonase has been observed in other plants. While PG is the main pectic enzyme observed in ripe tomatoes, in unripe green tomatoes, however, exopolygalacturonase is the main pectic enzyme observed (Pressey, 1987). Exopolygalacturonases have been reported in other fruits including peaches, pears, and apples (Pressey, 1987). The role of exopolygalacturonases is not well understood. Cell walls expand in growing plant tissue and the structural components must be modified to allow expansion and to accomodate newly synthesized components. Endohydrolases such as PG may be involved in these cell wall changes. In contrast, a role for exopolygalacturonase in pectin modification would involve only the monomers at the non-reducing ends of the galacturonan chains. Evidence has not been presented showing that such terminal residues have critical function in cell wall structure, but this cannot be ruled unlikely. A possibility is that terminal residues may be involved in calcium cross-links between adjacent chains. Such cross-links could be broken not only by removal of Ca^{+2} but also by hydrolysis of the glycosidic linkage of one of the residues by an exopolygalacturonase.

The oligogalacturonides observed in the untreated cotyledons are unlikely to be intermediates in cell wall biosynthesis. The biosynthesis of pectic polysaccharides has recently been investigated in sycamore maple suspension-cultured cells (Zhang and Staehelin, 1992). Immunolabeling patterns of suspension-cultured sycamore maple cells showed that the polygalacturonic acid (*i.e.*, larger than the oligogalacturonides) backbone is assembled in the cis- and medial cisternae of the golgi apparatus. Furthermore, methylesterification of the carboxyl groups of the galA occurs nearly simultaneous with polymerization in the golgi apparatus' medial cisternae. However, essentially nothing is known about the mechanism of chain termination in polysaccharide biosynthesis (Delmer and Stone, 1988). The mechanisms of transport of the biosynthesized polysaccharides,

their subsequent fusion to the plasma membrane, and their eventual deposition as part of the cell wall are not known either (Brett and Waldron, 1990).

The oligogalacturonide levels in the untreated cotyledons varied. In one set of experiments, the levels of oligogalacturonide observed in both untreated WbM (0.0) (resistant) and WbM (4.0) (susceptible) cotyledons were higher than those observed in mock- and *Xcm*-inoculated resistant and susceptible lines when IWF were prepared at 8 h and 17 h post-inoculation. In another set of experiments, the levels of oligogalacturonide observed were lower than those found in both mock- and *Xcm*-inoculated WbM (0.0) and WbM (4.0) cotyledons prepared at 4 h and 24 h post-inoculation. Possible reasons for such discrepancy are: 1) the age of the plants at the time IWF were collected. The much higher levels of oligogalacturonide observed were those from IWF collected from cotyledons 13 days after planting. The lower levels of oligogalacturonides in the untreated cotyledons were those from cotyledons when IWF were collected 15 days after planting. The rate of expansion or growth of a 15-day-old cotyledon is not as fast as in a 13-day-old cotyledon. The rate of turn-over and biosynthesis of the plant cell wall may also be slower at 15 days after planting thus a relatively lower level of non-covalently bound oligogalacturonide might be observed, 2) the time of day in which the IWF were collected. The relatively high levels of oligogalacturonide observed were those from IWF collected at 6.5 h into the photoperiod while the relatively lower levels were those from IWF prepared at 3.5 h into the photoperiod, and 3) it is possible that an uknown technical difficulty that is not controlled may have caused the differences in the values found.

The investigations conducted during these studies were directed toward testing the hypothesis that oligogalacturonides are endogenous elicitors of the plant defense response against the bacterial blight pathogen, *Xcm*. Because oligogalacturonides with dp = 11-13 are active in eliciting phytoalexin biosynthesis in cotton, elevated levels of these are expected during the incompatible interactions (resistant response) compared to the levels observed during the compatible interactions (susceptible response). The levels (pmol cm^{-2})

of the phytoalexin-elicitor-active oligogalacturonides found at various times are tabulated in Tables 10 and 11. The values for the decagalacturonide were also tabulated because it is active in eliciting phytoalexin biosynthesis in other systems (Davis *et al.*, 1986a). The treatments labeled *Xcm*-BH and mock-BH in both Tables 10 and 11 will be discussed later. The values in Table 10 show that levels of the phytoalexin-elicitor-active oligogalacturonides found in the incompatible interactions (resistant response) of WbM (0.0) plus *Xcm* rose to maximum at 8 h then fell between 8 and 17 h , but the rise and fall was not large between 4 h, 8 h, and 17 h post-inoculation. For example, the values found (pmol $cm^{-2} \pm$ standard error) for the dodecagalacturonide at 4 h, 8 h, and 17 h post-inoculation were 1.7± 0.6, 3.4 ± 1.2, and 2.7 ± 0.7, respectively. The levels found in the mock-inoculated samples were highest at 4 h post-inoculation, much lower at 8 h, and still lower at 17 h post-inoculation (Table 10). The levels of oligogalacturonides found in *Xcm*- and mock-inoculated WbM (0.0) were not significantly different at 8 h and 17 h post-inoculation. For example, the dodecagalacturonide levels found at 8 h post-inoculation were 3.4 ± 1.2 for the *Xcm*-treated sample and 3.3 ± 0.6 for the mock-inoculated sample. At 17 h post-inoculation, the values were 2.7 ± 0.7 and 1.8 ± 0.4 for the *Xcm*- and mock-inoculated samples respectively (Table 10). The IWF collected from mock-inoculated WbM (0.0) at 4 h post-inoculation had unusually high levels of oligogalacturonides. The PA-1 chromatogram of mock-inoculated WbM (0.0) at 4 h post-inoculation is shown in Figure 22. The values found were as high as the oligogalacturonide levels found in the untreated samples from this set of experiments. A possible reason for the high levels of oligogalacturonides found might be that the bacterial suspension medium (*i.e.*, water saturated with calcium carbonate) may have activated plant pectic enzyme. Plant exopolygalacturonases are known to be activated by Ca^{+2}. The solubility of calcium carbonate in water is 14 mg L^{-1} at 25°C (140 μM). The activity of the exopolygalacturonase from pear was increased 3-fold by addition of 500 μM Ca^{+2} to the reaction mixture (Pressey and Avants, 1976) while the activity of peach

exopolygalacturonase was increased 5-fold upon addition of 400 μM Ca^{+2} to the reaction mixture (Pressey and Avants, 1973). Although the activation of plant pectic enzymes is a formal possibility it is not a likely explanation because it did not appear to happen in the bacterially-treated samples or in other mock-inoculated controls.

The levels of elicitor-active oligogalacturonides found in the compatible interactions (susceptible response) of WbM (4.0) plus *Xcm* exhibited an increasing trend over 17 h (Table 11). For example, the values found for the dodecagalacturonide at 4 h, 8 h, and 17 h post-inoculation were 1.6, 4.7 ± 0.4, and 8.8 ± 1.6, respectively (Table 11). The levels found in the mock-inoculated samples of WbM (4.0) did not significantly change over 17 h (Table 11). Differences in the levels of oligogalacturonides were found between *Xcm*- and mock-inoculated WbM (4.0) cotyledons. For example, the levels of the dodecagalacturonide found at 8 h post-inoculation were 2.7 ± 0.1 in the mock-inoculated and 4.7 ± 0.4 in the *Xcm*-inoculated samples (Table 11). The levels of oligogalacturonides found at 17 h in *Xcm*-inoculated and mock-inoculated WbM (4.0) cotyledons are plotted in Figure 23. For all four oligogalacturonides plotted the values obtained from *Xcm*-inoculated samples were higher than those from mock-inoculated ones. This figure (Figure 23) also illustrates that of the four oligogalacturonides plotted, the levels were highest in the decagalacturonide, followed by the undecagalacturonide, then the dodecagalacturonide, and finally the tridecagalacturonide. This was generally the case for all samples, whether from *Xcm*- or mock-inoculated WbM (0.0) and WbM (4.0).

The levels of oligogalacturonides found in *Xcm*-inoculated resistant WbM (0.0) and susceptible WbM (4.0) lines were not significantly different from each other at 4 h and 8 h post-inoculation (Tables 10 and 11). However, the levels found at 17 h post-inoculation in the compatible interaction were higher than those found in the incompatible interaction. For example, the values found for the undecagalacturonide were 9.0 ± 1.0 for the compatible interaction and 3.1 ± 0.1 for the incompatible interaction.

The levels of oligogalacturonide observed at 4 h post-inoculation in both

incompatible and compatible interactions in the *Xcm*-treated cotyledons were lower than those found in the mock-inoculated cotyledons. Early during the interactions between the bacteria and the cotyledons, *Xcm* could be using the available oligogalacturonides as a source of nutrients. The catabolism of polygalacturonic acid by a pseudomonad has been investigated by Preiss and Ashwell (1963). A polygalacturonate-degrading enzyme preparation was partially purified from cell-free extracts of the pseudomonad and was shown to convert polygalacturonic acid to D-galA and 4-deoxy-L-threo-5-hexoseulose uronic acid, which were further metabolized.

The levels of oligogalacturonides found in both *Xcm*-inoculated WbM (0.0) (resistant) and WbM (4.0) (susceptible) cotyledons were normalized to the oligogalacturonide levels found in the mock-inoculated cotyledons of the same resistant or susceptible cotyledons for IWF collected at the same times after inoculation. These relative data are presented in Figure 24. The reason for this normalization were the following: 1) the levels observed in the uninoculated (untreated) cotyledons were the highest in one set of experiments and the lowest in the other set of experiments for those samples analyzed with an appropriate internal standard so relative values obtained, comparing between experiments, would be misleading, and 2) it is better to normalize oligogalacturonide levels found in the *Xcm*-treated cotyledons collected at a certain time to those observed from mock-inoculated samples collected at the same time after inoculation since the ages of the plants were the same at the time of IWF collection. The times of infiltration of the plants (*Xcm*- and mock-inoculated) and subsequent collection of IWF were only a few hours apart.

The levels of the undecagalacturonide and the dodecagalacturonide were chosen for comparison between treatments for the following reasons: 1) both oligogalacturonides are active not only in cotton but also in other plant systems in the elicitation of stress metabolite biosynthesis, and 2) the levels of both oligogalacturonides were as accurately quantitated by the method that was adopted (*i.e.*, PA-1 anion-exchange chromatography) as were dp =

8, 9, or 10. The tridecagalacturonide was not chosen because it was difficult to find a reproducible baseline for determining its peak area. When the levels of the undecagalcturonide and the dodecagalacturonide found in the *Xcm*-inoculated cotyledons were summed and normalized to those found in the mock-inoculated cotyledons the following ratios were obtained: 1) for the incompatible interactions (resistant response) of WbM (0.0) plus *Xcm*, the ratios obtained at 4 h, 8 h, and 17 h post-inoculation were 0.2, 1.0, and 1.2 respectively (Figure 24), and 2) for the compatible interactions (susceptible response) of WbM (4.0) plus *Xcm*, the ratio obtained at 4h, 8 h, and 17 h post-inoculation were 0.6, 1.9, and 2.7, respectively (Figure 24).

The results were contrary to the expectations; the relative levels of phytoalexin-elicitor-active oligogalacturonide found in the incompatible interactions were actually lower than those found in the compatible interactions (Figure 24). These results, however, could be reconciled to a number of observations previously made. Pierce (personal communication) found pectic enzyme activity (*i.e.*, likely PG though the possibility that it might be an exopolygalacturonase was not eliminated) from IWF prepared at 24 h post-inoculation only in the compatible interactions but not in incompatible interactions, using polygalacturonic acid as substrate. Since pectic enzyme is active in the IWF collected from compatible interactions, it is not surprising to see the presence of the oligogalacturonides. Since no pectic enzyme activity was observed in IWF obtained from incompatible interactions, that may explain why in this case the levels of oligogalacturonides did not change significantly over time. Venere and co-workers (1984) observed maximum pectic enzyme activity at pH 5.0 from homogenates at 24 h post-inoculation derived from a compatible *Xcm*-cotton interaction (*i.e.*, Ac 44E + *Xcm*). The enzyme preferred polygalacturonic acid as the substrate over citrus pectin. Very little pectic enzyme activity was observed from homogenates obtained from an incompatible interaction (*i.e.*, Im 216 + *Xcm*) assayed under the same conditions.

The hypothesis that oligogalacturonides are natural endogenous elicitors of the

defense response of cotton against *Xcm* cannot be ruled out even though elevated levels of oligogalacturonides were not observed during the incompatible interactions of WbM (0.0) plus *Xcm*. The levels of the elicitor-active oligogalacturonides available for extraction may not necessarily be elevated during incompatible interactions. It is possible that during the incompatible interactions of WbM (0.0) plus *Xcm* the phytoalexin-elicitor-active oligogalacturonides might have undergone a receptor-mediated endocytosis similar to that in the soybean suspension cultures described by Horn and co-workers (1989). They found that exogenous dodecagalacturonide was mostly internalized by soybean cell suspension cultures within 2 h.

An experiment that could be done to test the above hypothesis is the following: the dodecagalacturonide can be radioactively labeled as was described by Horn and co-workers (1989). A known amount of [^{125}I]-oligogalacturonide is incubated with known concentrations of cotton suspension cultures of both susceptible and resistant lines in the presence of *Xcm*. Aliquots can be withdrawn over a period of time, centrifuged to separate the cells (pellet containing bound or endocytosed dodecagalacturonide) from the medium (supernatant containing unbound dodecagalacturonide). The pellet is washed with 50 m*M* CDTA to eliminate the possibility of non-specific binding of the radioactively labeled dodecagalacturonide. Following the washing of the pellet, the radioactivity associated with both fractions is counted after homogenization of the pellet. A decrease in the radioactivity over time should be observed in the supernatant if a receptor-mediated endocytosis has occurred. Likewise an increase in radioactivity over time should be observed in the pellet. In the absence of receptor-mediated endocytosis, the radioactivity should be mostly associated with the supernatant. The experiment could also be accomplished using the method of Horn and co-workers (1989). The oligogalacturonide can be fluorescently labeled (with thiosemicarbazide) and incubated with cotton suspension cultures and *Xcm* as described above. Aliquots would be withdrawn at different times; phase contrast image of each aliquot taken, and then the fluorescent image of the same field

taken to show the location of the fluorescent elicitor. The above experiment assumes that *Xcm* will not metabolize the labeled dodecagalacturonide. The above experiment will apply to the first two possible scenarios described below and will not distinguish one from the other such that specific experiments may be necessary to distinguish one possibility from another. Variations of this method could also indicate the affinity or the number of receptors.

A possible scenario that may have led to the above process is the following: the product of an avirulence gene of *Xcm* may have directly or indirectly induced the activity of receptors in the resistant cotyledons but not in the susceptible cotyledons; that is, the product of the avirulence gene perhaps binds to a receptor in the resistant cotyledons which then influences the formation of the receptors for the oligogalacturonides. This may lead to differences in the rate of receptor-mediated endocytosis, synthesis of the receptor, and/or the affinity of the receptor.

The above scenario could lead to a difference in the number of receptors that bind the oligogalacturonides. In the case of the incompatible interactions (resistant response), there may have been a sufficient number of receptors for the oligogalacturonides that they were internalized fast enough that it was difficult to quantitate the changes in the levels of the oligogalacturonides. In the case of the compatible interactions, there may not be a sufficient number of receptors such that an apparent increase in the levels of oligogalacturonides could be observed over time.

Another explanation of the results is that during the compatible and incompatible interactions, the receptors may develop different affinities for the oligogalacturonides. It is possible that in the case of the incompatible interactions, the plant cells could be internalizing the oligogalacturonides through receptor-mediated endocytosis just as soon as the oligogalacturonides are produced. In the case of the compatible interactions (susceptible reaction), the affinity for the oligogalacturonides might be lower thus an increase over time is observed for the elicitor-active oligogalacturonides. In the case of the

compatible interactions, the receptors for the oligogalacturonides may have been desensitized (*i.e.*, a decrease in the response over time) so that the oligogalacturonides could not bind and therefore their levels increased over time. Other possible considerations are the state of phosphorylation of the receptors or their association with other proteins that are involved in the signal transduction process.

Another possible reason for the results is that elevated levels of oligogalacturonides may have occurred only around the bacterial microcolonies so that 'global differences' may be difficult to assess. But this is not likely since a change of levels of the oligogalacturonides was found during the compatible interactions (susceptible response) of WbM (4.0) plus *Xcm*.

Still another possibility is that the CDTA may have been such a good extractant that it obscured the real differences that might have been observed. For example, some of the oligogalacturonides observed in the CDTA extracts may have been the result of the disturbance of the normal cell wall structure. CDTA may have solubilized not only those oligogalacturonides resulting from *Xcm*-cotton interactions but also ones that are normally (hypothetically) associated with pectic 'egg-box' structure. If this was the case, an extracting solvent such as water might be better since it will not disturb the 'egg-box' structures.

Of course, it is possible that the original hypothesis that the oligogalacturonides are natural endogenous elicitors, is simply wrong.

Further improvement in the purification procedure might be warranted. This may include treatment of the IWF with a protease so that the possibility of reactivating a pectic enzyme is eliminated. In the purification scheme adopted, SDS is separated from the oligogalacturonides during the first chromatography step. SDS-inactivated pectic enzyme, however, elutes during the first chromatography along with the pooled fraction that contains oligogalacturonides. There is always a possibility that following removal of SDS, inactivated pectic enzymes could renature, resulting in the generation of oligogalacturonides

from the polymeric materials. During the second chromatographic purification, the supposedly SDS-inactivated pectic enzyme(s) is included in the fraction pooled for later derivatization with the 2-aminopyridine solution. SDS should be added during IWF collection to the solvent used for the first chromatography step (*i.e.*, HW40S). The IWF could also be treated with both SDS and mercaptoethanol at 37°C for 3 h to denature and reduce any pectic enzyme(s) present. Unusually high levels of oligogalacturonides observed in the mock-inoculated WbM (0.0) may have resulted from microbial contamination. Contamination may have occurred prior to SDS addition. A possible solution to the problem is to surface sterilize the cotyledons during harvest. This could be done by immersing the samples in a 1:5 dilution of commercial bleach. Another possible explanation to the unusually high levels observed in mock-inoculated WbM (0.0) is human error. It is possible that IWF may have been withdrawn for analysis from one of the untreated (uninoculated) IWF that contained relatively high levels of oligogalacturonides instead of from the correct vial.

Despite difficulties in explaining some results, particularly the unexpectedly high levels in some of the control samples, the method seems good since the replicates agree within 20% of each other even though analyses of replicates were in each case performed a month apart. The precision of the analytical method was tested. The coefficients of variation (CV) between replicates for all samples were calculated and averaged. The following average CVs (± standard deviation) were obtained: CV = 18.1% ± 3.7 (n = 14) when the levels of oligogalacturonides found (pmol cm^{-2}) with dp = 9-12 were summed up for each replicate and the CV between the two replicates was calculated; CV = 20.2% ± 4.4 (n = 14) when oligogalacturonides with dp = 8-12 were summed up; CV = 21.6% ± 3.9 (n = 14) for dp = 8 only; CV= 16.9% ± 3.5 (n = 14) for dp = 9 only; CV = 17.9% ± 3.6 (n = 14) for dp = 10 only; CV = 20.6% ± 5.2 (n = 14) for dp = 11 only; CV = 20.2% ± 4.4 (n = 14) for dp = 12 only. A non-parametric method of statistical evaluation was also made. The CVs (n = 70) between replicates for individual oligogalacturonides dp = 8-12 were

ranked, and analysis of variance on those values was made to test if the CVs observed between oligogalacturonides with different dp could be judged to be the same. The test was conducted by the statistics department. Results of the analysis of variance gave a P = 0.9697. The value was interpreted by the statistician, Dr. M. Payton as follows: there was very strong evidence to support the conclusion that the CVs seen from one oligogalacturonide to another oligogalacturonide were the same. It would be almost impossible to find values that were more alike since the P value was almost equal to 1.00. In summary, the coefficients of variation between replicates were close to 20% for all treatments and for all dp's evaluated.

A number of intercellular wash fluids from various interactions were subjected to the demethylesterification procedure described in the experimental section prior to dialysis and subsequent quantitation of oligogalacturonide levels by PA-1 anion-exchange chromatography. This procedure was prompted by two reports indicating that enzymes with different substrate preference are activated during the incompatible and compatible interactions between *Xcm* and cotton (Hopper *et al.*, 1975; Venere *et al.*, 1984). First, Hopper and co-workers (1975) found pectic enzyme activity from sterile culture filtrates of *Xcm* grown on an autoclaved cotton leaf medium. The culture filtrates digested citrus pectin, suggesting a preference for a highly-esterified substrate. Second, Venere and co-workers (1984) observed that pectic enzyme activities were detected early (~3 h post-inoculation) from homogenates obtained from *Xcm*-inoculated cotyledons of the highly resistant line Im 216. These workers observed maximum pectic enzyme activity when extracts were assayed at pH 5 rather than at pH 7. The pectic enzyme detected preferred citrus pectin over polygalacturonic acid as substrate. The pectic enzyme activities measured from the compatible interactions were observed later in the interactions and preferred polygalacturonic acid as substrate. If pectic enzyme activity with a preference for an esterified substrate is observed in *Xcm*-cotton interactions, then products that are esterified should also be produced. IWF were saponified for 3 h at pH 11.5, at which conditions

methyl-esterified galacturonic acid residues are deesterified (see Figure 25). Samples that were subjected to saponification are labeled with BH after the designation *Xcm*- or mock- in Tables 10 and 11.

During the incompatible interactions between WbM (0.0) plus *Xcm*, the levels of oligogalacturonides found in saponified IWF were higher relative to the levels found in unsaponified IWF. For example, at 4 h post-inoculation, the levels of the dodecagalacturonide were 1.7 ± 0.6 for the unsaponified IWF and 3.5 ± 0.0 for the saponified sample (Table 10). Elevated oligogalacturonide levels were observed in other oligogalacturonides as well following deesterification. At 4 h post-inoculation, the level of the decagalacturonide increased 2.3-fold (*i.e.*, from 3.7 pmol cm^{-2} to 8.4 pmol cm^{-2}) after subjecting the IWF to the deesterification procedure. During the compatible interactions between WbM (4.0) plus *Xcm*, the levels of oligogalacturonides were lower in the saponified IWF than they were in the unsaponified IWF. For example the levels of the dodecagalacturonide at 8 h post-inoculation were 4.7 ± 0.4 in the unsaponified IWF and 3.4 ± 0.3 in the saponified IWF (Table 11).

The levels of the elicitor-active undecagalacturonide plus dodecagalacturonide observed from a replicate of a particular sample after the demethylesterification procedure were normalized to the levels of the undecagalacturonide plus dodecagalacturonide observed in the same replicate sample that was not subjected to the saponification procedure. The following results were found: 1) ratios observed in the incompatible interactions [WbM (0.0) + *Xcm*] were the following: 2.3 at 4 h post-inoculation, 2.0 at 8 h post-inoculation, and 2.1 at 17 h post-inoculation (Figure 25), 2) the following ratios were observed in the compatible interactions [WbM (4.0) + *Xcm*] were the following: 0.6 at 8 h post-inoculation, and 0.5 at 17 h post-inoculation (Figure 25), 3) ratios from mock-inoculated WbM (0.0) and WbM (4.0) were less than or equal to one (see Tables 10 and 11). The results of the saponfication of IWF from the mock-inoculated and compatible interactions will be addressed later.

Oligogalacturonides that were fully or partially esterified appear to have been generated during the incompatible interactions between *Xcm* and the resistant line WbM (0.0). It appears that the levels generated were approximately equal to the levels of unesterified oligogalacturonide already present because an apparent doubling in the levels of the elicitor-active undecagalacturonide and the dodecagalacturonide after the saponification of the IWF was observed at all time points examined (see Figure 25). Further, these results are concordant with the finding of Venere and co-workers (1984) that a pectic enzyme with a preference for an esterified substrate is the predominant enzyme expressed during incompatible interactions. The observation that a corresponding increase after the deesterification procedure in the levels of the undecagalacturonide and the dodecagalacturonide did not occur in the mock-inoculated WbM (0.0) appears to indicate that the fragments were perhaps generated by a pectic enzyme of bacterial origin or that a pectic enzyme of plant origin with preference for esterified substrates may have been activated only in the incompatible (resistant) interactions. The most probable origin of the esterified oligogalacturonides generated during the incompatible interaction between *Xcm* and WbM (0.0) is the homogalacturonan region of the cotyledon cell wall or middle lamella. Mort and co-workers have examined the DOM of the homogalacturonan region of 14-day-old cotyledons of Ac 44E. Their results indicated that the homogalacturonan region of the cotyledon is at least 50% methyl-esterified (DOM = 50%) (Mort, personal communication). At this time, it is not clear how the methyl groups are distributed in the cotyledons' homogalacturonan region.

No corresponding increases were detected in the compatible interactions [*i.e.*, WbM (4.0) + *Xcm*]. This finding appears to suggest that a pectic enzyme that prefers an esterified substrate is not active in the compatible interactions during at least the first 17 h period. The pectic enzyme that may have generated the esterified oligogalacturonide fragments during the incompatible interactions between *Xcm* and the resistant cotton cotyledons of WbM (0.0) is probably the endopolymethylgalacturonase (PMG). Like PG,

PMG has the same enzyme commission number of 3.2.1.15 (Venere *et al.*, 1984). These enzymes are differentiated only by their substrate preference; the PG prefers polygalacturonic acid while PMG prefers citrus pectin.

A number of reports have documented the presence of the pectic enzyme PMG and its relation to pathogenicity has been investigated. Kim and co-workers (1990) observed considerable amounts of PMG activities in two isolates of *Penicillium expansum.* In apple medium, the activities of PMG and PG of the strongly pathogenic isolate were over six times higher than those of the weakly pathogenic isolate. Damn and Strzelczyk (1987) found that all isolates of *Cylindrocarpon destructans* pathogenic to fir and pine produced pectic enzyme activities. However, there was no correlation found between PMG and pathogenicity. Seventeen of 19 isolates that were pathogenic produced PMG activities while 9 of 11 isolates that were not pathogenic to both fir and pine produced PMG activity.

The deesterification procedure was designed to convert both partially-esterified and/or fully-esterified oligoglacturonides to fully-deesterified oligogalacturonides (Figure 25). Possible sources of the observed increase in levels of fully deesterified oligogalacturonides are : 1) fully-deesterified oligogalacturonides, and 2) deesterified oligogalacturonides derived from partially- and fully-esterified oligogalacturonides. The levels of oligogalacturonide were expected to remain constant after saponification if oligogalacturonides that were partially- or fully-esterified were absent in the IWF. However, decreases in the oligogalacturonide levels were observed in the susceptible interactions and in the mock-inoculated IWF samples. These decreases could be explained by the following: at higher pH (*i.e.*, pH 11.50), the open linear forms at the reducing ends of the oligogalacturonides could undergo a couple of reactions. The carbonyl carbon at the reducing end of the molecule could undergo oxidation and would thus be converted to a carboxylic acid group, or the carbonyl carbon at the reducing end could be reduced and thus converted to an alcohol group. When either reaction occurs, the products could no longer couple with the 2-aminopyridine reagent and therefore, no glycosylamine derivative

could be produced, thus a decrease in the amount of the oligogalacturonide would be observed. In order to calculate a decrease in the levels of undecagalacturonide and dodecagalacturonide after the deesterification reaction, then a smaller decrease in the internal standard must have occurred. If the decrease in oligogalacturonide levels in the compatible [WbM (4.0) + *Xcm*] interaction could be explained by oxidation/reduction at pH 11.5, then the same process(es) might have occurred in the IWF collected from incompatible interactions [WbM (0.0) + *Xcm*]. One possible explanation is the amounts of partially-esterified or fully-esterified oligogalacturonides were so substantial that the deesterified products formed after the deesterification reaction could have masked the probable effects (*i.e.*, oxidation/reduction of the carbonyl carbon at the reducing end of the molecule) of subjecting the oligogalacturonides to pH 11.50. If that scenario occurred, then the increase in the levels of oligogalacturonides after deesterification observed from the incompatible interactions are actually higher than calculated. An experiment was performed to find out if the undecagalacturonide is more susceptible to degradation at pH 11.50 than the internal standard. The internal standard and the undecagalacturonide were incubated together and subjected to the saponification procedure. The mixture was derivatized with 2-aminopyridine and chromatographed on a PA-1 column. The ratio of the peak areas of the undecagalacturonide to the internal standard was compared to the ratio of the peak areas of the undecagalacturonide to the internal standard (control) that did not undergo a saponification procedure. The ratios were the same indicating that the undecagalacturonide was not more susceptible to degradation by base than the internal standard. Furthermore, neither oligomer appeared to have been degraded.

A different explanation can be postulated to explain the decrease observed in oligogalacturonides from mock-inoculated and compatible interactions following deesterification: it is possible that compounds such as high mol wt methyl-esterified oligogalacturonides co-chromatographed with molecules of interest prior to saponification. If for example a peak observed was a 50:50 mixture of the high mol wt oligogalacturonide

and the molecule of interest, then saponification would result in a 50% decrease in the level of the oligogalacturonide observed. After deesterification, the high mol wt oligogalacturonide may not have been observed because in was retained longer by the PA-1 column. This may have happened also to the IWF derived from incompatible interactions except that the amount of methyl-esterified oligogalacturonides generated were so substantial that the apparent decrease was not observed.

Methyl-esterified oligogalacturonides appear to have been generated during the incompatible interactions between cotton and *Xcm*. It remains to be investigated whether the actual elicitor-active signal molecules that bind to plant receptors might be the methyl-esterified oligogalacturonides. The phytoalexin-elicitor activity of methyl-esterified oligogalacturonides merits further investigation. A number of reports appear to indicate that methyl-esterified oligogalacturonides might be more active than the less-esterified oligogalacturonides in eliciting phytoalexin production. Komae and co-workers (1990) found that *awkeotsang* oligogalacturonides derived from *awkeotsang* polygalacturonide (DOM = 62%) were more active in the soybean cotyledon bioassay than oligogalacturonides derived from low methyl-esterified apple pectin (DOM = 29.8%), when both polygalacturonides were treated with the PL from *Erwinia carotovora*. Their evidence, however, is indirect since the actual chemical structure(s) of the active molecule(s) was not investigated further. Nothnagel and co-workers (1983) also observed activity in the soybean cotyledon bioassay of crude pectic fragments generated from acid hydrolysis of citrus pectin obtained from Sigma Chemicals. When citrus pectin-derived fragments were subjected to anion-exchange chromatography on QAE-Sephadex, early-eluting fractions were collected, pooled, and analyzed for sugar composition. The fraction contained 90% neutral sugars and ~10% uronic acid by weight. Since the fraction represented carbohydrate molecules not retained by the QAE-Sephadex anion-exchange column, the uronic acid moieties were most likely partially- or fully-methyl-esterified. Although this fraction had a low uronic acid content (~10% by wt), its activity in the

soybean cotyledon bioassay was ~1/2 the activity observed for the uronic acid-rich fraction (>90% uronic acid by wt), a pooled fraction that was highly retained by the anion-exchange column, when the fractions were applied to the cotyledons at the same total carbohydrate wt per cotyledon. No further characterization of the fraction was done, however. Davis and co-workers (1984) observed that pectic fragments generated by treating citrus pectin with PL from *Erwinia carotovora* were active in the soybean cotyledon bioassay. The chemical nature of the fragments was not investigated. Amin and co-workers (1986) reported that heat stable elicitors were released from citrus pectin which then elicited the synthesis of the phytoalexin 6-methoxymellein after incubation of citrus pectin with carrot suspension cultures in the presence of culture filtrates of *Chaetomium globosum*. Elicitation was specific for citrus pectin since control samples did not elicit 6-methoxymellein production. The nature of the pectic fragments released from citrus pectin was not characterized further. Another report of possible elicitor activity of methyl-esterified oligogalacturonides was obtained by Jin and West (1984). These workers methylated an oligogalacturonide mixture (*i.e.*, dp = 12-15) with diazomethane. Apparently, the fully-methylated oligogalacturonide mixture had 1/20 the activity of the fully deesterified oligogalacturonide mixture in the castor bean bioassay although they reported variability in the response. In two of the cases, however, Jin and West (1984) reported that the fully methyl-esterified oligogalacturonide mixture appeared to have the same activity as the non-derivatized oligogalacturonide mixture. Furthermore, demethylation of the fully-methylated oligogalacturonide mixture resulted in a product that was more active than the non-derivatized oligogalacturonide mixture, suggesting that the deesterification reaction may have been incomplete and that one or more methyl groups not removed may have resulted in a molecule whose elicitor-activity is greater than the non-derivatized sample. One way of testing whether methyl-esterified oligogalacturonides are active in eliciting phytoalexins is to synthesize these molecules chemically.

Methyl-esterified oligogalacturonides appear to be more stable than fully

deesterified oligogalacturonides towards enzyme degradation. Davis and co-workers (1984) reported that heat-stable elicitors released from citrus pectin were more stable to degradation by the PL from *Erwinia carotovora* than elicitors released from sodium polypectate. The use of methyl-esterified oligogalacturonides as elicitors in the defense against potential pathogens might be advantageous to plants. Once digested out of the homogalacturonan region, methyl-esterified oligogalacturonides are there to be active and are available to be receptor-bound. They are unlikely to form the 'egg-box' structures coordinated by Ca^{+2} that fully-deesterified oligogalacturonides may.

It is also worth investigating whether a different complement of pectic enzymes might be active during the two types of interactions (*i.e.*, compatible and incompatible interactions). For example, is PME inactive during incompatible interactions (resistant response) such that oligogalacturonides that are methyl-esterified are produced? PME activity has been observed a number of times in culture filtrates of *Xcm* as described in the review of literature. Is PME active in the compatible interactions (susceptible response) such that methyl-esterified oligogalacturonides that are produced are immediately deesterified, resulting in a less active elicitor molecule such as the fully deesterified oligogalacturonides? An an answer to those questions can perhaps be addressed by measuring PME activity in IWF collected from both incompatible and compatible interactions.

Another observation that warrants further investigation into the elicitor activity of methyl-esterified oligogalacturonides is that relatively high concentrations of exogenous deesterified oligogalacturonides are required for them to serve as elicitors in the assay systems examined to date. Perhaps further chemical characterization and determination of structural requirements for elicitor-activity of the oligogalacturonides are needed.

Albershiem and co-workers (1992) have found six modified oligogalacturonides in the tridecagalacturonide-enriched fraction (obtained by digestion of polygalacturonic acid with PG) during anion-exchange chromatography. The modified oligogalacturonides

accounted for 15% of the tridecagalacturonide-rich fraction. Albersheim and co-workers (1992) also mentioned that modified oligogalacturonides were also present in all of the conventional ion-exchange fractions containing biologically-active oligogalacturonides (*i.e.*, dp = 10-15). The modified oligogalacturonides, however, appeared to have little or no biological activity.

Calculations were made to determine whether high enough oligogalacturonide quantities were present in IWF to trigger phytoalexin biosynthesis. The highest levels of oligogalacturonides were found in the untreated 13-day-old WbM (4.0) (susceptible) cotton cotyledons and were used for sample calculations. The sum of oligogalacturonides with dp = 10-13 was 74 pmol cm^{-2}. The value was corrected for the percent efficiency of the extraction from the cotyledons (*i.e.*, 74 pmol cm^{-2} x 1/0.70 = 106 pmol cm^{-2}). The sum of oligogalacturonides with dp = 10-13 was converted to pg cm^{-2} by multiplying the calculated value in pmol cm^{-2} with the average mol wt of the phytoalexin-elicitor-active oligogalacturonides (*i.e.*, ~2000 pg $pmol^{-1}$). The value obtained was 0.21 μg cm^{-2}. It was compared to the concentration of dodecagalacturonide (in μg cm^{-2}) that reproducibly elicited phytoalexin biosynthesis in OK 1.2 cotyledons (*i.e.*, 4.9 μg cm^{-2} plant tissue). The value of 4.9 μg cm^{-2} was obtained by multiplying the concentration of the dodecagalacturonide that reproducibly elicited phytoalexin elicitation (0.546 mg mL^{-1}) by the value obtained for the intercellular space (*i.e.*, ~9 μL cm^{-2}). When amounts are compared (in μg cm^{-2}), the concentration of the elicitor-active oligogalacturonides found in WbM (4.0) cotyledons was 1/23 of the dodecagalacturonide that reproducibly triggered the biosynthesis of phytoalexins in OK 1.2 cotyledons.

Since the average concentration (in pmol cm^{-2}) of phytoalexin-elicitor-active oligogalacturonides (*i.e.*, dp = 10-13) was determined, calculations were made to find out what percent of available substrate is in the elicitor-active form. In order to calculate that value, the concentration of galA in μmol cm^{-2} plant tissue must be known. This value is ~1.61 μmol galA (monomeric form) cm^{-2} cotton cotyledon and was determined by Mort

and Grover (1988). The level of galA (monomeric form) that can be derived from the phytoalexin-elicitor-active oligogalacturonides was calculated to be 814 pmol cm^{-2} by assuming that the elicitor-active oligogalacturonides have an average dp of 11 [*i.e.*, 74 pmol 11-mer x 11 pmol galA monomer / pmol 11-mer = 814 pmol galA monomer cm^{-2}]. The value of 814 pmol cm^{-2} represents ~0.05% of the available substrate. Thus, an estimated 0.05% of the available substrate was in the form of elicitor-active oligogalacturonides. The ~0.05% of the available substrate in the form of elicitor-active oligogalacturonides might be in the right range for a signal molecule.

CHAPTER V

CONCLUSIONS

The following conclusions can be derived from the investigations conducted:

1) Oligogalacturonides elicit phytoalexin biosynthesis in cotton cotyledons. However, the amount of oligogalacturonide required to reproducibly elicit phytoalexin biosynthesis was relatively high. A concentration of ~0.50 mg mL^{-1} of the tridecagalacturonide produced half-maximal response. Phytoalexin levels fluctuated during the period of accumulation; therefore, harvesting at several times would be the best way to assess whether phytoalexins have accumulated.

2) It is possible to elute oligogalacturonides from the cell wall matrix, purify them from other components of intercellular wash fluids, and quantitate them individually in the size range of oligomers known to be phytoalexin-elicitor-active. The method developed between replicates agreed within 20% of each other. Nevertheless, further improvement in the purification procedure might be warranted. As was discussed in the preceding section, this may include treatment of the IWF with a protease, or addition of SDS during the first chromatography step. Both treatments would help eliminate possible pectic enzyme activity. Finally, surface sterilization of the collected samples would also help eliminate microbial contamination. Improvement in the method of quantitating oligogalacturonides may include capillary gel electrophoresis, with which Liu and co-workers (1992) were able to detect oligogalacturonide derivatives in the low attomole (10^{-18} mol) range.

3) Oligogalacturonides, including those that are phytoalexin-elicitor-active, are present when IWF are collected from resistant and susceptible cotton cotyledons inoculated

with *Xcm*, mock-inoculated, or uninoculated. Their presence in the untreated (uninoculated) cotyledons could result during expansion growth of the plant cell wall. Their abundance in cotton cotyledons represents roughly ~0.05% of the galA in the mesophyll cell walls of cotton cotyledons. This is the first documented demonstration of the *in vivo* presence of oligogalacturonides.

An experiment is recommended to address the relationship between rate of cotyledon expansion and levels of oligogalacturonides. This was prompted by the difference in the levels of oligogalacturonides observed in uninoculated cotyledon between 13 day- and 15 day-old plants. IWF can be collected at different stages of growth from untreated (uninoculated) cotyledons and analyzed for oligogalacturonides. It would be the first report on the relationship between cotyledon growth and oligogalacturonide levels.

4) Results of the experiments do not support the hypothesis tested since elevated levels of non-esterified oligogalacturonides were observed in the susceptible cotyledons but not in the resistant cotyledons after bacterial inoculation. However, the hypothesis could not be ruled out since other factors might be involved as was discussed earlier. The presence in untreated cotyledons of non-esterified oligogalacturonides, including those that are phytoalexin-elicitor-active casts some doubt about their direct involvement in the resistant response of cotton against the bacterial blight pathogen, *Xcm*. If they are signal molecules in the resistant response leading to phytoalexin production, their absolute levels in IWF do not reflect it. It is not likely that an answer regarding their role in resistance can be obtained from quantitative analysis of non-esterified oligogalacturonides in IWF.

5) An increasing trend over time in the non-esterified oligogalacturonide levels observed from compatible interactions [*Xcm* + WbM (4.0)] appears to indicate that a pectic enzyme which prefers a non-esterified substrate might be active during the interactions of the bacterial blight pathogen and the susceptible cotyledons.

6) The elevation in levels of oligogalacturonides observed at all times after saponification of the IWF specifically from incompatible interactions [*i.e.*, *Xcm* + WbM

(0.0)] indicates that methyl-esterified oligogalacturonides were generated. The observation indirectly indicates that an enzyme which prefers methyl-esterified substrates might have been active. Further studies are needed to investigate whether there is a correlation between expression of this enzyme and the induction of the defense response of cotton against the bacterial blight pathogen, *Xcm*. Also, further studies are needed to investigate whether there is a correlation between production of methyl-esterified oligogalacturonides and the induction of the defense response.

Experiments are recommended to analyze IWF from incompatible interactions for the presence of methyl-esterified oligogalacturonides. IWF can be purified through PA-1 anion-exchange chromatography with no saponification. Fractions co-chromatographing with the neutral molecules, as well as those co-chromatographing with the oligogalacturonides, can be collected separately, dried, and subjected to saponification. If subsequent chromatography on PA-1 of the saponified fraction that co-chromatographed with the neutral molecules shows the presence of peaks chromatographing at the retention times of standard deesterified oligogalacturonides, this would be an indication of the presence of methyl-esterified oligogalacturonides in the original PA-1 fraction collected. If PA-1 chromatography following saponification of oligogalacturonide fractions collected shows a peak of the original oligogalacturonide plus a peak(s) eluting later, this would also indicate the presence in IWF of methyl-esterified oligogalacturonides. Further chemical characterization of the fractions might be warranted. This may include mass spectral analysis of the oligogalacturonide fractions.

Chemical synthesis of methyl-esterified oligogalacturonides would be helpful. It would allow testing of phytoalexin-eliciting activity of a pure material, valuable tests not just in cotton but in other systems as well, especially in systems where indirect eveidence suggests activity of methyl-esterified oligogalacturonides.

CHAPTER VI

EXPERIMENTAL

Reagents

CDTA (*trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid hydrate), 2-aminopyridine, sodium azide, 2,2,4-trimethylpentane (isooctane), methanol, *n*-butanol, methyl acetate, pyridine, and imidazole were all reagent grade and purchased from Aldrich Chemical Company (Milwaukee, WI). Ammonium acetate (>99%) was purchased from Fluka Chemicals (Fluka Chemie, Switzerland). Sodium dodecyl sulfate (SDS) was an electrophoresis purity reagent from Bio-Rad Laboratories (Richmond, CA). Potassium oxalate was reagent grade and obtained from Mallinckrodt Chemical Company (St. Louis, MO). Tri-Sil Concentrate, a mixture of hexamethydisilazane and trimethylchlorosilane, was purchased from Sigma Chemical Company (St. Louis, MO). An *E. coli* clone (HB101/pAKC213:Tn5-2) expressing the *peh* gene from *Erwinia carotovora* sbsp. *carotovora* was the source of the endopolygalacturonase (PG) and was kindly provided by Dr. A. K. Chatterjee (University of Missouri, Columbia, MO). Storage and purification of the endopolygalacturonase by Dr. Maness was the same as that described by Maness and Mort (1989). The internal standard used, the oligogalacturonide with dp = 6 produced by treating polygalacturonic acid with endopectate lyase, was a kind gift from Dr. A. Hotchkiss (USDA, ARS, Philadelphia, PA).

Bacterial Cultures

Bacteria

The *Xanthomonas campestris* pv. *malvacearum* strain 3631 used in these studies was a single-colony isolate from a spontaneous streptomycin-resistant mutant of the highly aggressive race 3 field isolate from Altus, OK, that was previously described by Essenberg and co-workers (Essenberg *et al.*, 1982). Maintenance and culture were by R.A. Samad essentially as described by Pierce and Essenberg (1987). Bacteria were amplified in 50 mL nutrient broth in a 125-mL flask with a side arm at 30°C for about 15 h until an optical density of between 0.12 and 0.18 was reached. The absorbance was measured with a Coleman Junior spectrophotometer (Coleman Instruments Inc., Maywood, IL). The concentrated culture was collected by centrifugation at full speed (1700*g*) on a bench-top centrifuge for 3 min and resuspended in 15 mL of 14% (v/v) glycerol and 86% (v/v) nutrient broth (Difco Laboratories, Detroit, MI). The bacterial inoculum was dispensed in 1.5-mL Eppendorf tubes at 1.0 mL per tube, followed by freezing with dry ice/acetone. The bacterial cultures were subsequently stored in a -70°C freezer until further use.

Preparation of Bacterial Inocula

When needed, a scoop of the frozen bacterial culture was added to sterile nutrient broth. Bacterial cultures were amplified on a gyrotory shaker rotated at 190 rpm (New Brunswick Scientific Inc., New Brunswick, NJ) for about 18 hours in a 30°C walk-in incubator (Percival Instruments, Boone, IA) until an optical density between 0.12 and 0.18 was reached. The bacterial cultures were divided and transferred to sterile 12-mL conical centrifuge tubes and spun down at full speed (1700*g*) on an bench-top centrifuge for 5 min. The nutrient broth was decanted and the actively growing bacteria were resuspended in an equal volume of sterile water saturated with calcium carbonate (0.14 mg mL^{-1}). A known volume of the concentrated bacterial suspension was withdrawn and diluted with a calcium

carbonate-saturated water solution until an approximate inoculum concentration of 1-2 x 10^8 colony-forming units (cfu) mL^{-1} was attained. Serial dilutions of the bacterial inoculum were made with water and the final dilution was plated on a Difco nutrient agar plate containing 200 ppm streptomycin sulfate with the use of a spiral plater (Spiral Systems Inc., Cincinnati, OH). The agar plates were turned upside down 45 min after plating and placed in a plastic wrapper. The agar plates containing the bacterial colonies were incubated in the 30°C walk-in incubator for 2 days and the number of yellow bacterial colonies counted shortly thereafter.

Plant Materials

Cotton Lines

Acala 44 (Ac44) possesses no major genes for resistance to *X. campestris* pv. *malvaceaurum* and is susceptible to all known races of this pathogen (Essenberg *et al.*, 1982). The cotton line OK 1.2 (Pierce and Essenberg, 1987) was derived from segregating generations of a cross between the blight-susceptible line Ac44 and the blight-immune line Immune 216 (Im 216). Im 216 possesses homozygous resistance, that is thought to include the major resistance genes B_2, B_3, and b_7 on a polygenic background (Brinkerhoff *et al.*, 1984). It is immune to bacterial blight; *i. e.*, it is resistant to all known races of *X. campestris* pv. *malvacearum* and shows no macroscopically visible symptoms of natural infection under field conditions (Brinkerhoff *et al.*, 1984).

Near-isogenic blight-resistant WbM (0.0) and blight susceptible WbM (4.0) lines were derived from resistant cultivar Westburn M, which was developed by the Oklahoma Agricultural Experiment Station by a complex series of crosses and selections. The resistance gene content of WbM (0.0) has not yet been determined, but probably it includes most if not all of the genes present in Im 216, as well as possibly B_N (Essenberg *et al.*, 1982).

Growth of Cotton Plants

Seeds were sown in 15-cm diameter clay pots (at least 12 seeds per pot) containing Jiffy Mix-Plus (Jiffy Products of America, West Chicago, IL). Two days before an experiment, plants were thinned to between 6 to 8 plants per 15-cm pot. Plants were grown as described by Pierce and co-workers (1992) in Conviron E15 growth chambers set to provide a regimen consisting of a 14-h light period and 10-h dark period. The middle 10 h of the photoperiod was at the highest photosynthetic photon flux density, which averaged 5 x 10^2 $\mu mol \cdot s^{-1}$ m^{-2} at plant height. Light intensity increased and decreased in stages (four stages for both the incandescent and fluorescent lamps) during the 2-h dawn and dusk periods. The temperature during the high-light period was 30°C and 19°C during the middle 8 h of the dark period.

Inoculation and Mock-inoculation of Cotton Cotyledons

Infiltration of cotyledons with the bacterial inocula was the same as that described by Pierce and Essenberg (1987). Cotton cotyledons, which in cotton are leaf-like, were inoculated 13-14 d after planting. Inoculation was done in the middle of the photoperiod. Pots were taken out of the growth chamber ~2.5 h before infiltration and put back in the growth chamber after the water of infiltration had transpired (*i.e.*, ~60 min). The cotton cotyledon abaxial epidermis was pricked with a needle in four places. Inoculum was applied through the needle pricks with the use of a 1-mL tuberculin syringe (no needle) (Becton-Dickinson & Co., Rutherford, N.J.) until the entire intercellular space was filled. Ocassionally, extra pricks (*i.e.*, one or two) were needed to obtain complete infiltration of the intercellular space. All plants within one pot (*i.e.*, 6-8 plants) received the same treatment. In general, pots of susceptible and resistant cotton plants getting the same treatment were treated alternately.

Phytoalexin Elicitation with Purified Oligogalacturonides

For the elicitation experiments using purified oligogalacturonides, the same infiltration procedure was used as described earlier except that only the middle two sections of the cotyledon were infiltrated through pricks near the base. Infiltrations were done near the middle of the photoperiod. The oligogalacturonide fraction 375 used was obtained by autoclave-hydrolysis of polygalacturonic acid and was a gift from Dr. A. J. Mort (Department of Biochemistry, Oklahoma State University). It was obtained by eluting the autoclave-hydrolysates of polygalacturonic acid from the DEAE column with 375 m*M* potassium chloride buffered at pH 5.2, thus name fraction 375 (Maness *et al.*, 1991). The undecagalacturonide, dodecagalacturonide, and tridecagalacturonide were purified from autoclave-hydrolysate of polygalacturonic acid as described by Jin and West (Jin and West, 1984) and were a gift from Dr. M. L. Pierce (Department of Biochemistry, Oklahoma State University). Sample discs were collected with the use of sterile cork borers at a distance from the needle prick (*i.e.*, the point of infiltration). Each sample disc was 0.60 cm^2 for both the dodecagalacturonide and tridecagalacturonide experiments. Each sample disc for the oligogalacturonide fraction 375 experiments was 0.40 cm^2. Collected samples were wrapped in an aluminum foil, frozen in liquid nitrogen, and stored immediately in a -70°C freezer.

Analytical Procedures

Preparation of 50 m*M* CDTA Solution

CDTA (10.4 g) was added to 500 mL of water in a 1-L beaker. The suspension was stirred slowly. The pH of the suspension was slowly adjusted to 6.50 by carefully adding a 10 *N* solution of sodium hydroxide a few drops at a time. As the pH approached 6.50, a 1*N* NaOH solution was used instead. Upon reaching pH 6.50, the solution was diluted to 600 mL. The solution was filtered (HA 0.45 μm filter, Millipore Corporation,

Bedford, MA) with the use of a water aspirator and stored in the refrigerator until use.

Preparation of Intercellular Wash Fluids (IWF)

Thirty to forty half cotton cotyledons (equivalent to a replicate) were placed in an Erlenmeyer flask (125 mL or 250 mL) containing 80-120 mL of the extracting solvent. Vacuum was applied for 3 min through a water aspirator. Release of vacuum caused infiltration of the majority of the cotyledons' intercellular spaces by the extracting solvent. Infiltration of the cotyledons was completed by 10-sec applications and then release of the vacuum 2-3 times.

The infiltrated cotyledons were removed from the erlenmeyer flask with the use of large tweezers, carefully to avoid tissue damage. The infiltrated cotyledons were transferred to paper towels and carefully blotted with Kimwipes. Samples were subsequently stacked in groups of 12-14 half-cotyledons. Each stack was placed in a rectangular polypropylene insert that when rolled to touch opposing edges fitted the circumference of the IWF collection tubes. The wrapped cotyledons were placed in the IWF collection tubes with the cotyledons supported vertically (cut edge down) on a grid above the bottom of the tube. At the bottom of the tube was added 10 µL of 10% (w/v) SDS.

The infiltrated tissue was centrifuged at 3400 rpm (1200*g*) for 30 min in a SS34 rotor. At the end of the centrifugation, the cotyledons were removed carefully with the use of tweezers and wrapped in aluminum foil for later determination of total surface area. The intercellular wash fluids were transfered to sterile eppendorf tubes (capacity 1.5 mL) with the use of a sterile pasteur pipette. Each IWF collection tube was rinsed with ~0.5 mL sterile water and the water rinse added to the collected IWF. The IWFs were spun at 12,000 rpm for 5 min in an Eppendorf centrifuge 5415 (Brinkman Laboratories) at 4°C in order to remove the bacteria and the bacterial lipopolysaccharide. The supernatant was withdrawn with a disposable sterile needle (25G 3/8, Becton-Dickinson & Co.,

Rutherford, NJ) attached to a sterile tuberculin syringe (1 mL, Becton-Dickinson & Co., Rutherford, NJ). The needle was removed and replaced with a 0.45-μm filter (Cole-Parmer Instrument Co., Chicago, IL). The sample was filtered gently and collected in a clean sterile container and its volume measured by weighing. Whenever necessary, SDS was added such that its final concentration was ~0.25% (w/v). IWF was stored in a -70°C freezer until further analysis by gel permeation chromatography. In general, each sample required ~2.5 h to collect the IWF. Total surface area of the cotyledons was determined using a Model LI-3000 Li-cor portable area meter (Lambda Instrument Corp., Milwaukee, WI) which was made available for use by Dr. B. Martin of the Department of Agronomy.

Gel Permeation Chromatography on HW40S

Chromatography was performed on a SP8700 HPLC system (Spectra-Physics, San Jose, CA). A volume of IWF collected equivalent to 50-80 cm^2 of plant material was withdrawn, and an internal standard was added (dp = 6, derived from treating polygalacturonic acid with endopectate lyase). In general, 4.50 μg (4.20 nmol) of hexagalacturonide internal standard was added to each sample. The sample, a 0.8 mL-1.0 mL aliquot of IWF, was injected onto a preparative HPLC column (50 cm x 22.5 mm, 1" o.d.) packed with TSK-Gel Toyopearl HW40S (Supelco Inc., Bellefonte, PA) after filtration through a 0.45 μm filter unit (0.45 μm hydrophilic filter unit, Cole-Parmer Instrument Company, Chicago, IL). The Toyopearl HW40S resins are made up of synthetic methacrylic polymers with a particle size range of 20-40 μm and are able to fractionate dextrans from 100-7,000 mol wt. The solvent used was a 200 m*M* solution of ammonium acetate buffered at pH 5.20 and delivered at 2.0 mL min^{-1}. The compounds eluting off the column were detected by a Waters differential refractometer model R401 set at 8x and collected in two fractions: molecules that eluted close to the void volume, V_o, (~22-36 ± 1 min) and molecules that eluted near the included volume, V_i, (~36-54 min). The fractions were collected in 50-mL Erlenmeyer flasks that had been silanized. Fractions

were collected manually as they were exiting the refractive index detector without additional tubing. The differential refractometers used for the experiments were loaned to us by Dr. C. Adams (Chemistry Department) and the Department of Foods and Nutrition. The first fraction contained the oligogalacturonides plus polymeric materials. The oligogalacturonide-containing fraction (*i.e*, molecules that eluted near the V_o) was concentrated through the use of a *Speed Vac* (Savant Instruments, Inc., Farmingdale, NY). Prior to concentration in the *Speed Vac*, the sample was transferred to five 8-mL vials that had been silanized. Samples were concentrated on the *Speed Vac* overnight. Water (2 mL) was added to the last of the 5 vials and vortexed thoroughly. The solution was carefully transferred to the fourth vial and thorough mixing was repeated. The process was repeated from the fourth vial to the third vial and so on, until the 2-mL solution reached the first collection vial. The entire process was repeated twice but these times only 1.5 mL of water was used instead of 2 mL. At the end of the transfers approximately 5 mL of water had been transferred from the last vial to the first vial through the three intermediate vials. The sample was concentrated again as before until the volume was suitable for the second gel permeation chromatography step.

The concentrated or dried samples were reconstituted or diluted to ~0.80 mL with sterile water and filtered through a 0.45 μm sterile filter unit (0.45 μm hydrophilic filter unit, Cole-Parmer Instrument Company, Chicago, IL) before the gel permeation chromatography on a TSK2000SW column.

For the recovery studies with the undecagalacturonide-rich fraction, a volume of IWF collected equivalent to 15 cm^2 of plant tissue was withdrawn and injected on a semi-preparative column (500 mm x 10 mm, 0.5" o.d.) packed with TSK-Gel Toyopearl HW40S. The solvent used was a 200 m*M* ammonium acetate buffered at pH 5.20 and delivered at 0.5 mL min^{-1}. The fraction that co-eluted with and near the V_o, which included the undecagalacturonide, was collected and dried as before prior to the second gel-permeation chromatography step.

During the recovery studies with the undecagalacturonide-rich fraction, the CDTA extracts were obtained from WbM (0.0) represnting 22 cotyledons and harvested from 20 cotton plants. Of the cotyledons used for these experiments, only the middle part of each cotyledon was treated (*i.e.*, the areas adjacent to the midvein). Once harvested, the midvein of each cotyledon was cut off plus the untreated regions resulting in two triangular-like pieces. One of the two pieces was mixed with halves from the other 21 cotyledons to represent a replicate. The other halves represented the second replicate. The control samples, treated with water only, consisted of 25 cotyledons from 20 cotton plants. Each replicate was obtained by the same procedure described above for the undecagalacturonide-treated cotyledons. The undecagalacturonide-treated and the control cotyledons to be extracted with either imidazole or water represented material taken from 10 different cotton plants. The manner in which the two replicates were obtained was essentially the same as that described above for extraction with CDTA.

Gel Permeation Chromatography on TSK2000SW

Chromatography was performed on a SP8700 HPLC system (Spectra Physics, San Jose, CA). Compounds that eluted close to the V_0 during the HW40S GPC chromatography, which included the oligogalacturonides, were divided into two fractions by chromatography on Spherogel-TSK 2000SW GPC column (Altex Inc., 10 μm, 7.5 mm i.d. x 30 cm). A 200 m*M* ammonium acetate solution buffered at pH 5.20 was used as the eluant and pumped at 0.5 mL min^{-1}. The first fraction consisted of compounds that eluted between 8-14 min and co-chromatographed with the V_0. The second fraction, which contained the oligogalacturonides, consisted of compounds that eluted between 14-24 min. A Waters differential refractometer model R401 set at 8x was used for detection. The two fractions, each contained in two 8-mL silanized vials were concentrated to dryness overnight with the use of the *Speed Vac* concentrator. Whenever possible, the pooled fraction eluting off the TSK2000SW column between 14-24 min post-injection was dried

immediately otherwise it was covered with parafilm and stored at -20°C. The dried samples were reconstituted in water to a volume of 3 mL prior to dialysis or for some IWF samples, prior to deesterification at pH 11.50.

Dialysis of Samples Prior to Derivatization and PA-1 Anion-exchange Chromatography

Dialysis was performed using 1000 mol wt cut-off cellulose ester dialysis membranes (Spectra Por 6, Spectrum Medical Industries Inc., Houston,TX) as described by Mort and co-workers (Mort *et al.*, 1991). All dialysis membranes used were pre-wetted and supplied in 0.05% (w/v) sodium azide. Membranes were washed with sterile water prior to dialysis. Samples containing the oligogalacturonides from the TSK2000SW GPC were dissolved in at least 3 mL of distilled water and dialyzed overnight against water in a 4-L flask or beaker at 4°C with constant stirring. No more than eight samples were dialyzed together. Samples were dialyzed between 16-18 h, with one change of water after 6 h. At the end of the dialysis, samples contained in the membranes were transferred to a suitable silanized container. The dialysis membrane was washed with another 2 mL of water and the rinse was combined with samples and dried in the *Speed Vac* concentrator. The dried samples were redissolved in 0.7 mL water and transferred to a micro-reaction vessel (*ca.* 1 mL) and dried again in the *Speed Vac* . The original container for each sample was rinsed with another 0.7 mL water and the solution transferred to the corresponding micro-reaction vessel. The solution was again dried with the use of the *Speed Vac.*

Preparation of Pyridylamino Oligosaccharide Derivatives

The preparation of oligosaccharide derivatives was as described by Maness and Mort (1991) with minor modifications. Samples were derivatized in micro-reaction vessels (1 mL, Supelco Inc., Bellefonte, PA) in 25 µL aqueous 2-aminopyridine. The vials were sealed securely with Teflon-lined caps, and the reaction mixtures were incubated at 65 °C between 12-15 h. The 2-aminopyridine derivatizing solution was 4.0 *M*. Typically,

between 5-6 g of 2-aminopyridine was dissolved in 5 mL water with gentle stirring. The pH was slowly adjusted to 7.00 with glacial acetic acid. Upon reaching pH 7.00, the solution was diluted with water to the appropriate final volume (~7.5 mL) in a graduated cylinder. The 2-aminopyridine derivatizing mixture was stored cold in an amber colored container under an argon or nitrogen atmosphere to prevent decomposition.

Purification of Pyridylamino Oligosaccharide Derivatives

Cation-exchange Extract-Clean columns (100 mg packing size, exchange capacity of 0.2 to 0.3 meq g^{-1}) were obtained from Alltech (Alltech Associates Inc., Deerfield, IL). Columns were preconditioned with concentrated ammonia (*ca.* 10 mL) and then rinsed with water (*ca.* 20 mL) until the pH was between 5-6. Samples that had been derivatized with the 2-aminopyridine solution were diluted to 20 times their original volume with the addition of 0.44 *M* acetic acid (*i.e.*, by the addition of 380 μL of the 0.44 *M* acetic acid solution). The diluted derivative was applied to the pre-conditioned Extract-Clean column using a pasteur pipette. The eluate was collected in a test tube. The vial was rinsed with two 200-μL portions of water and the rinsings were applied to the Extract-Clean column and eluates were collected, combined, and mixed prior to PA-1 anion-exchange chromatography.

PA-1 Anion-exchange Chromatography

Anion-exchange chromatography of the derivatized oligogalacturonides was similar to that described by Hotchkiss and Hicks (1990) with minor modifications. Separations were performed using an SP8700 HPLC system (Spectra-Physics, San Jose, CA). Samples were chromatographed on a PA-1 Carbopac anion-exchange column (4 x 250 mm, Dionex Corp., CA) fitted with a Carbopac PA guard column (3 x 25 mm, Dionex Corp., CA). The Carbopac PA-1 anion-exchange column is made up of 10 μm polystyrene-divinylbenzene backbone agglomerated with 350 nm microbead quaternary

amine functionalized latex. The solvent system consisted of 500 m*M* potassium oxalate solution buffered at pH 7.8 in reservoir A and water in reservoir B. Initially, the ionic strength of the system was 0.080 (*i.e.*, 25 m*M* or 5% A and 95% B). The initial condition was maintained during the first minute, after which the ionic strength of the system was linearly increased to 0.30 (*i.e.*, 100 m*M* potassium oxalate or 20% A and 80% B) over 9 min, and then linearly increased to 275 m*M* oxalate over 45 min (*i.e.*, 55% A and 45% B). The flow rate was held constant throughout at 0.7 mL min^{-1}. Upon reaching 55% A, the liquid chromatograph was programmed to go back to initial conditions over 5 min. Another 10 min was allowed to pass before a new injection was made. Derivatized molecules eluting from the column were detected by their fluorescence using a RF-535 fluorescence HPLC monitor fitted with a xenon arc-lamp (Shimadzu Inc., Kyoto, Japan). Derivatized molecules were excited at 290 nm and fluorescence intensity was monitored at 360 nm. Data analysis was performed by a Macintosh IIx computer using the A.C. Chrom alias program (Merz and Mort, 1992).

Alternatively, a sodium acetate (unadjusted) gradient was used instead of potassium oxalate and was similar to that described by Maness and Mort (1989) with minor modifications. Samples were chromatographed on a PA-1 Carbopac anion-exchange column as described above. The solvent system consisted of 200 m*M* sodium acetate in reservoir A and 900 m*M* sodium acetate in reservoir B. Initially, the ionic strength of the system was at 0.550 (*i.e.*, 550 m*M* sodium acetate or 50% A and 50% B). The initial condition was maintained during the next 10 min, after which the ionic strength of the system was linearly increased to 0.760 over 50 min (*i.e.*, 760 m*M* sodium acetate or 20% A and 80% B). The flow rate was held constant throughout at 1.0 mL min^{-1}. Upon reaching 20% A and 80% B, the chromatographic conditions were maintained during the next 25 min, and then programmed to go back to the initial conditions over 15 min. Using the sodium acetate gradient, shorter oligogalacturonides (*i.e.*, dp = 1-4) were eluted during the first 10 min. Oligogalacturonides of medium sizes (*i.e.*, dp = 5 to dp = 11) were eluted

using a linear gradient from 550 m*M* to 760 m*M* in 50 min. Oligogalacturonides with dp = 12-16 were eluted within the next 25 min after the ionic strength of 760 m*M* has been reached. Another 15 min was allowed to pass before a new injection was made. Detection and quantitation of the oligogalacturonides were the same as described above.

Anion-Exchange Chromatography on TSK DEAE-2SW

Anion-exchange chromatography on TSK DEAE-2SW of the glycosylamine derivatives was similar to that described by Maness and Mort (1989) with few modifications. Separations were performed using a Beckman Model 334 gradient liquid chromatograph (Beckman Instruments Inc., San Ramon, CA). The system consisted of a Model 421A system controller, two Model 110B high-pressure pumps, a Model 210A sample injector and a Model 163 variable wavelength detector. The solvent system consisted of 10% methanol in 30 m*M* sodium acetate, pH 5.2, in reservoir A and 500 m*M* sodium acetate, pH 5.2, in reservoir B. Oligogalacturonides were separated on a Spherogel TSK DEAE-2SW analytical anion-exchange column (4.6 x 200 mm; Beckman Instruments, Inc., San Ramon, CA) using a flow rate of 1.0 mL min^{-1} at 35°C. Initially the ionic strength of the system was at 0.032 (*i.e.*, 32 m*M* sodium acetate or 90% A and 10% B). The initial condition was maintained during the next 10 min, after which the ionic strength was linearly increased 0.232 over a 30 min period (*i.e.*, 232 m*M* sodium acetate or 57% A and 43% B). This condition was maintained for the next 50 min after which the gradient was programmed to back to the initial conditions over 5 min.

Base Hydrolysis of Methyl Esterified Oligogalacturonides

A number of samples from the TSK2000SW chromatography were subjected to a demethylesterification procedure. Samples were dissolved in 3 mL of sterile distilled water. The pH of the solution was adjusted to 11.50 with 1.0 *N* NaOH. The solution was stirred at 4°C. The pH of each sample was checked at the end of 1 h. If the pH had dipped

below 11, the pH was readjusted back to 11.50 by adding 0.1 *N* NaOH. The reaction was allowed to proceed for 3 h. Dialysis and PA-1 anion-exchange chromatography of these samples were the same as described above.

Methanolysis and Derivatization of Sugars

Trimethylsilyl methyl glycosides were prepared by the method of Chaplin with minor modification (Chaplin, 1982; Komalavilas and Mort, 1989). A known volume of sample was withdrawn and added to a vial containing 100 nmol of inositol as internal standard. The solvent was evaporated with the use of the *Speed Vac*. Methanolysis was performed as follows: 250 μL of 1.5 *M* methanolic HCl was added to the sample and 50 μL of methyl acetate was added to protect the reaction mixture from water. The samples were tightly cappped with caps lined with Teflon-rubber laminated discs (Pierce Chemical Co., Rockford, IL) and placed in a heated block at 80°C. Caps were tightened again after 10 min to prevent leakage. Methanolysis was allowed to proceed for 13-15 h, and then the reaction mixture was allowed to cool down to room temperature. Eight drops of *n*-butanol were added to each vial and the samples were evaporated under a stream of nitrogen gas. During drying of the samples, the trimethylsilylating agent was prepared fresh by mixing 1 volume of Tri-Sil concentrate (Pierce Chemical Co., Rockford, IL) and 3 volumes of pyridine. Fifty microliters of the reagent was added to each nitrogen-dried sample. The vials were capped quickly and shaken gently to make sure that the derivatizing reagent contacted the entire internal surface. The samples were allowed to derivatize for 15 min at room temperature. The excess derivatizing agent was evaporated carefully under a stream of argon and the samples were redissolved in 10-20 μL isooctane. The samples were injected onto a gas chromatograph within 30 min of derivatization.

Gas-liquid Chromatography (GLC) of Sugar Derivatives

One to two microliters of the derivatized sugars were injected into a fused silica

capillary column and followed with 1 μL isooctane. The trimethylsilyl methyl glycosides were chromatographed (Komalavilas and Mort, 1989) by GLC using a Durabond-1 capillary column (DB-1, 30 m x 0.25 mm i.d., 25 μm film thickness, J & W Scientific Inc., Rancho Cordova, CA) installed in a Varian 3300 gas-liquid chromatograph equipped with an on-column injector and helium carrier gas. The sample was injected carefully, maintained at 105°C for 1 min, then raised to 160°C at a rate of 10°C min^{-1} and held for 4 min, before being raised again at a rate of 2°C min^{-1} to 210°C with no hold. Upon reaching 210°C, the temperature was raised to 240°C at 10°C min^{-1} and maintained at that temperature for 10 minutes. Total run time was 48.50 min. Alternatively, a longer temperature program (Pierce *et al.*, 1992) was employed in a number of samples. The sample was injected at 105°C and maintained at that temperature for 1 min. The temperature was raised to 160°C at 10°C min^{-1} and maintained at that temperature for 4 min. The temperature was again raised to 170°C at a rate of 0.5°C min^{-1} with no hold. The temperature was immediately raised to 204°C at a rate of 2°C min^{-1} with no hold. Upon reaching 204°C, the temperature was again raised to 230°C at 10°C min^{-1} and maintained at that temperature for 20 min. The total run time was 70.10 min. Peak integration was performed using a Varian 4290 integrator. Data analysis was performed on a Macintosh microcomputer using a Microsoft Excel spreadsheet program (Mort, unpublished).

Extraction of Phytoalexins

Plant tissue of known surface area was extracted three times in a glass homogenizer with with 16 mL of 80% (v/v) methanol per gram fresh weight. If tissue was less than 0.06 g in weight, 1 mL of 80% methanol was used regardless of the weight. Following each homogenization, the homogenate was centrifuged at top speed for 3 min (1700*g*) in a bench-top centrifuge. The combined extracts were taken to aqueous after adding 1 mL water with the use of an Evapo-mix system (~10 min). The resulting solution was passed

through a Sep-Pak C_{18} cartridge (Waters Associates, Millford, MA) that had been equilibrated with 2.0 mL methanol and then 5.0 mL water prior to the introduction of the sample. The Evapo-mix tube and then the Sep-Pak were rinsed with 2 mL of water, then with 2 mL 30% (v/v) methanol. The phytoalexins were eluted with 6.0 mL 85% methanol and collected in a conical centrifuge tube. Water was added to dilute the eluate to 40% methanol, and the sample was partitioned two times with volumes of chloroform equal to one-half the volume of 40% methanol. The chloroform extracts were taken to dryness with a stream of nitrogen. Extracted phytoalexins were stored dry under nitrogen in the freezer at -70°C until quantitation by high performance liquid chromatography.

HPLC of Phytoalexins

Chemical analysis of phytoalexins was similar to that described by Pierce and Essenberg with some minor modifications (Pierce and Essenberg, 1987). Samples for analysis were taken up in 300 µL of 60% methanol (v/v) and chromatographed by HPLC on an SP8700 HPLC system (Spectra-Physics, San Jose, CA) fitted with a Hibar LiChrosorb RP-18 (5 mm, 250 mm x 4 mm i.d., E. Merck, Darmstadt, Germany) along with a Hibar pre-packed column and a Waters model 440 absorbance detector (λ = 254 nm). The phytoalexins were eluted from the column with the following solvent program: isocratic at 60% methanol from 0 to 20 min at 1 mL min^{-1}, a linear gradient to 67% methanol from 20 to 25 min at 1.5 mL min^{-1}, isocratic thereafter at 67% methanol at 1.5 mL min^{-1}. Fractions were collected on the basis of signals from an ISCO model 2150 peak separator (ISCO, Lincoln, NE). The levels of phytoalexins plotted have already been adjusted to the percent recovery of each stress metabolite: 97% for LC, 98% for DHC, and 80% for HMC.

Incubations with the Endopolygalacturonase (PG) from *Erwinia carotovora*

Aliquots of standard undecagalacturonide and IWF collected from *Xcm*-infiltrated WbM 0.0 cotton cotyledons that had undergone purification through HW40S and TSK2000SW gel permeation chromatography steps were withdrawn and dried in the *Speed Vac*. Once dried, the standard undecagalacturonide and IWF from *Xcm*-infiltrated cotton cotyledons were redissolved in 0.5 mL of 70 m*M* ammonium acetate buffered at pH 5.5. To each solution was added 0.1 unit of PG acivity and the reaction mixture incubated for 30 min at room temperature. At the end of 30 min, the solutions were dried and glycosylamine derivatives were prepared by the addition of 20 μL of the 2-aminopyridine reagent. The control solutions were the same amount IWF collected from *Xcm*-infiltrated cotton cotyledons and the same amount of standard undecagalacturonide solutions dissolved in 0.5 mL ammonium acetate buffer at pH 5.5 allowed to stand for 30 min without the PG and derivatized with 20 μL of the 2-aminopyridine reagent. Derivatization and work-up of the derivatized oligogalacturonides were the same as described earlier. The pectic nature of the peaks were judged by whether the peaks observed in the controls have dissappeared in the reaction mixtures treated with the PG.

Determination of Intercellular Volume

Cotton cotyledons were grown from resistant WbM (0.0) or resistant OK 1.2 seeds. Twelve-d-old, fourteen-d-old, or seventeen-d-old cotyledons were infiltrated with a known inoculum of *Xcm* (~4.5 x 10^6 cfu mL^{-1}) close to the middle of the photoperiod. Samples (0.40-1.20 cm^2) were excised with the use of sterile cork borer from the infiltrated cotyledons either immediately after inoculation or after the water of infiltration had transpired. It took ~70 min for complete transpiration of the water of infiltration. After excision, samples were thoroughly washed twice with sterile water (~15 mL each time), blotted dry with Kimwipes, and immediately homogenized in a sterile mortar and pestle.

Typically, 1.20 cm^2 of plant tissue was homogenized in 16 mL sterile saturated $CaCO_3$ solution after washing (1 mL first and then 15 mL). The homogenized sample was plated using a spiral plater onto a nutrient agar as was the diluted original *Xcm* inoculum. The spiral plated samples were incubated in the 30°C incubator for 2 days and bacterial colonies were counted shortly thereafter. The value of intercellular volume in $\mu L\ cm^{-2}$ was computed by dividing the number of cfu cm^{-2} found in the excised samples by the number of cfu μL^{-1} in the original inoculum.

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APPENDIXES

APPENDIX A

TABLES

TABLE 1

STRESS METABOLITES ELICITED IN RESISTANT OK 1.2 COTTON COTYLEDONS 40 HOURS AFTER INFILTRATION WITH VARIOUS CONCENTRATIONS OF THE DODECAGALACTURONIDE

	Stress Metabolites Elicited (nmol cm^{-2})[a] ± Standard Error			
Dodecagalacturonide Concentration (mg mL^{-1})	Total Stress Metabolites	LC	DHC	HMC
0.000	0.10 ± 0.10	0.05 ± 0.02	0.05 ± 0.02	0.00 ± 0.00
0.009	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.017	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
0.034	0.28 ± 0.00	0.15 ± 0.10	0.13 ± 0.11	0.00 ± 0.00
0.068	1.2 ± 0.7	0.45 ± 0.39	0.68 ± 0.68	0.07 ± 0.07
0.137	0.74 ± 0.74	0.33 ± 0.33	0.41 ± 0.41	0.00 ± 0.00
0.273	1.2 ± 0.3	0.18 ± 0.17	0.25 ± 0.12	0.76 ± 0.19
0.546	7.3 ± 2.4	0.23 ± 0.14	1.6 ± 0.5	5.5 ± 1.9

[a]Values reported were the average of three replicates. Each replicate represents 10 discs (6 cm^2) harvested from 10 different cotyledons and pooled. The experiment was done once.

TABLE 2

STRESS METABOLITES ELICITED IN SUSCEPTIBLE ACALA 44E AND RESISTANT OK 1.2 COTTON COTYLEDONS 22 HOURS AFTER INFILTRATION WITH VARIOUS CONCENTRATIONS OF THE TRIDECAGALACTURONIDE

	Stress Metabolites Elicited (nmol cm^{-2})[a] in Ac 44E			
Concentration (mg mL^{-1}) Tridecagalacturonide	Total Stress Metabolites	LC	DHC	HMC
0.00	0.00	0.00	0.00	0.00
0.25	0.20	0.02	0.04	0.17
0.50	0.90	0.08	0.11	0.72
1.00	2.5	0.74	0.69	1.1
1.50	1.4	0.14	0.11	1.1

	Stress Metabolites Elicited (nmol cm^{-2})[b] in OK 1.2			
Concentration (mg mL^{-1}) Tridecagalacturonide	Total Stress Metabolites	LC	DHC	HMC
0.00	0.02	0.00	0.01	0.01
0.25	0.07	0.00	0.07	0.00
0.50	0.10	0.01	0.03	0.07
1.00	1.8	0.15	0.27	1.4
1.50	1.2	0.14	0.26	0.81

Table 2 (Continued)

[a]Values reported were from five discs (3.0 cm^2) harvested from five cotyledons, pooled, and analyzed for stress metabolites. The experiment was done once.

[b]Values reported were from three discs (1.8 cm^2) harvested from three cotyledons, pooled, and analyzed for stress metabolites. The experiment was done once.

TABLE 3

STRESS METABOLITES ELICITED IN SUSCEPTIBLE ACALA 44E AND RESISTANT OK 1.2 COTTON COTYLEDONS 40 HOURS AFTER INFILTRATION WITH VARIOUS CONCENTRATIONS OF THE TRIDECAGALACTURONIDE

	Stress Metabolites Elicited (nmol cm^{-2})[a] in Acala 44E			
Concentration (mg mL^{-1}) Tridecagalacturonide	Total Stress Metabolites	LC	DHC	HMC
0.00	0.08	0.00	0.08	0.00
0.25	0.20	0.01	0.09	0.12
0.50	1.3	0.07	0.18	1.0
1.00	2.1	0.26	1.3	0.49
1.50	6.3	0.62	1.6	4.1

	Stress Metabolites Elicited (nmol cm^{-2})[b] in OK 1.2			
Concentration (mg mL^{-1}) Tridecagalacturonide	Total Stress Metabolites	LC	DHC	HMC
0.00	0.03	0.00	0.00	0.03
0.25	0.07	0.00	0.00	0.07
0.50	10	2.3	4.8	3.0
1.00	17	4.6	7.8	4.8
1.50	16	1.6	5.1	8.9

Table 3 (Continued)

[a]Values reported were from five discs (3.0 cm^2) harvested from five cotyledons, pooled, and analyzed for stress metabolites. The experiment was done once.

[b]Values reported were from three discs (1.8 cm^2) harvested from three cotyledons, pooled, and analyzed for stress metabolites. The experiment was done once.

TABLE 4

SUGAR COMPOSITIONS OF IWF PREPARED AT DIFFERENT TIMES FROM 14-DAY-OLD OK 1.2 RESISTANT COTTON COTYLEDONS INOCULATED WITH *Xcm* (1.71×10^8 cfu mL^{-1}) AND FROM SUSCEPTIBLE ACALA 44E COTTON COTYLEDONS INOCULATED WITH *Xcm* (1.66×10^8 cfu mL^{-1})

Sugar Levels[a] (nmol cm^{-2}) in IWF[b] from OK 1.2					
Time (h)	Ara	Gal	GalA	Rha	Xyl
0.5	2.6 ± 0.1	3.9 ± 0.1	0.26 ± 0.02	0.33 ± 0.01	1.3 ± 0.1
4	1.6 ± 0.4	2.6 ± 0.7	0.33 ± 0.22	0.25 ± 0.07	0.83 ± 0.09
8	2.7 ± 0.6	4.1 ± 0.8	1.2 ± 0.4	0.38 ± 0.06	1.2 ± 0.1
12	2.5 ± 0.4	4.0 ± 0.6	0.75 ± 0.18	0.34 ± 0.00	1.1 ± 0.2
16	3.1 ± 1.1	4.9 ± 1.6	0.52 ± 0.36	0.31 ± 0.00	1.1 ± 0.3
20	2.8 ± 0.7	4.2 ± 0.8	0.28 ± 0.02	0.36 ± 0.05	0.64 ± 0.09
24	5.5 ± 1.5	7.6 ± 2.2	0.33 ± 0.07	0.62 ± 0.20	0.80 ± 0.24
Sugar Levels[a] (nmol cm^{-2}) in IWF[b] from Acala 44E					
Time (h)	Ara	Gal	GalA	Rha	Xyl
1	2.1 ± 0.7	3.2 ± 0.8	0.24 ± 0.04	0.34 ± 0.05	1.3 ± 0.1
4	2.9 ± 0.7	4.5 ± 0.3	0.43 ± 0.08	0.40 ± 0.04	1.3 ± 0.3
8	2.8 ± 0.5	4.6 ± 0.5	1.1 ± 0.2	0.41 ± 0.05	0.70 ± 0.24
12	2.8	4.5	1.0	0.45	1.1
16	3.3 ± 0.1	5.6 ± 0.1	1.1 ± 0.0	0.52 ± 0.00	1.1 ± 0.0
20	3.7 ± 0.7	6.3 ± 0.6	1.1 ± 0.3	0.61 ± 0.06	1.3 ± 0.06
24	2.0 ± 0.4	4.8 ± 0.5	0.94 ± 0.26	0.89 ± 0.02	0.40 ± 0.03

Table 4 (Continued)

[a]Values reported were the average of two replicates ± standard error. Values from the Ac 44E IWF at 12 h post-inoculation were from one replicate.

[b]IWF was dialyzed (1000 mol wt cut-off) for ~16 h against water.

TABLE 5

GALACTURONIC ACID LEVELS OF UNTREATED, WATER-TREATED, AND MOCK-INOCULATED 14- OR 15-DAY-OLD COTTON COTYLEDONS EXTRACTED WITH VARIOUS SOLVENTS WHEN IWF WERE PREPARED AT DIFFERENT TIMES AFTER INFILTRATION

Cotton Line	Treatment[a]	Solvent	Galacturonic Acid (nmol cm^{-2})
WbM (0.0)	Untreated	CDTA	12[b], 14[b]
WbM (0.0)	Water-treated	Imidazole	0.24, 0.30
WbM (4.0)	Water-treated	Imidazole	0.19, 0.38
WbM (0.0)	Water-treated	Water	0.22
WbM (4.0)	Water-treated	Water	0.22
WbM (0.0)	Mock-inoculated	CDTA	
	1st Extract		24[c]
	2nd Extract		29[c]
WbM (0.0)	Water-treated	Imidazole	
	1st Extract		0.29, 0.00
	2nd Extract		0.00, 0.00
WbM (4.0)	Water-treated	Imidazole	
	1st Extract		0.09, 0.15
	2nd Extract		0.00, 0.09
Ac 44E	Untreated	Water	0.51, 0.96
Ac 44E	Mock-inoculated	Water	0.27, 0.16
Ac 44E	Mock-inoculated	Water	0.57[d], 0.66[d]
OK 1.2	Untreated	Water	0.11, 0.53
OK 1.2	Mock-inoculated	Water	0.15, 0.03
OK 1.2	Mock-inoculated	Water	0.48[d], 0.35[d]

[a]IWF were prepared immediately after the water of infiltration had transpired, unless indicated otherwise.

Table 5 (Continued)

[b]Galacturonic acid levels when 1st and 2nd extracts were combined.
[c]Galacturonic acid levels when IWF were collected 8 h after mock-inoculation.
[d]Galacturonic acid levels when IWF were collected 24 h after mock-inoculation.

TABLE 6

DETERMINATION OF INTERCELLULAR VOLUME IN MICROLITERS PER SQUARE CENTIMETER OF TWELVE- TO SEVENTEEN-DAY-OLD OK 1.2 AND WESTBURN M (0.0) COTTON COTYLEDONS

Cotton Line	Age of Cotyledons (d)	Area Excised (cm^2)	Intercellular Volume[a] ($\mu L\ cm^{-2}$) ± Standard Error
WbM (0.0)	14	1.23	8.9 ± 0.7[b]
WbM (0.0)	17	0.41	12.0 ± 1.2[c]
OK 1.2	12	0.82	13.8 ± 0.4[c]
OK 1.2	14	0.82	12.4 ± 0.8[c]

[a]Values were derived from four replicates. Each replicate was spiral-plated twice.

[b]Value obtained when sample discs from *Xcm*-inoculated plant tissue were excised immediately, homogenized, and spiral-plated within 40 min.

[c]Value obtained when the water of infiltration was allowed to transpire (*ca.* 70 min) from *Xcm*-inoculated cotyledons before samples were excised, homogenized, and plated.

TABLE 7

SUMMARY OF THE UNDECAGALACTURONIDE RECOVERY STUDIES. FOURTEEN-DAY-OLD RESISTANT COTTON COTYLEDONS OF WbM (0.0) WERE INFILTRATED WITH UNDECAGALACTURONIDE[a] AND IWF WERE PREPARED IMMEDIATELY AFTER THE WATER OF INFILTRATION HAD TRANSPIRED

Extracting Medium	% Recovery of Each Extract		% Total Recovery
	1st Extract	2nd Extract	
Water	15.0	1.0	16.0
CDTA 50 m*M*, pH 6.5	63.0	8.0	71.0
	58.0	12.0	70.0
Imidazole 500 m*M*, pH 7.0	2.0	6.0	8.0

[a]The concentration of the undecagalacturonide-rich fraction applied was 0.5 mg mL^{-1}.

TABLE 8

ESTIMATED RECOVERY OF OLIGOGALACTURONIDES EXTRACTED WITH CDTA FROM COTYLEDONS AND PURIFIED BY GEL PERMEATION CHROMATOGRAPHY

Step	Recovery for Given Step[a] (%)	Sequential Recovery (%)
CDTA Extraction	70	70
HW40S Chromatography	70	49
TSK2000SW Chromatography	50	25

[a]Value for CDTA was from the recovery studies with the undecagalacturonide. Values for the HW40S chromatography and TSK2000SW chromatography were from recovery studies with the dodecagalacturonide.

TABLE 9

LEVELS OF PHYTOALEXIN-ELICITOR-ACTIVE OLIGOGALACTURONIDES PRESENT IN UNTREATED (UNINOCULATED) WESTBURN (0.0) AND WESTBURN (4.0) COTTON COTYLEDONS WHEN IWF WERE PREPARED USING CDTA AS EXTRACTANT

DP Oligogalacturonide	Levels Found (pmol cm^{-2})[a]		
	WbM (0.0)[b]	WbM (4.0)[b]	WbM (0.0)[c]
10	24.4 ± 1.2	32.7 ± 1.4	5.6
11	13.6 ± 2.1	19.6 ± 1.4	2.1
12	8.4 ± 2.1	12.6 ± 0.8	2.0
13	5.4 ± 0.5	10.1 ± 1.9	0.7

[a]Values presented were the average of two replicates ± standard error. Values from the 15-day-old cotyledons were from one replicate; the second replicate could not be accurately quantitated because PL product dp = 5 was used as internal standard at too low a level. No values were reported from 15-day-old WbM (4.0) because sample was lost during dialysis.

[b]Values reported were from 13-day-old cotyledons.

[c]Values reported were from 15-day-old cotyledons.

TABLE 10

LEVELS OF PHYTOALEXIN-ELICITOR-ACTIVE OLIGOGALACTURONIDES FOUND IN IWF AT VARIOUS TIMES FROM *Xcm*- AND MOCK-INOCULATED FOURTEEN-DAY-OLD RESISTANT WESTBURN (0.0) COTTON COTYLEDONS USING CDTA AS EXTRACTANT

		Levels Found (pmol cm^{-2})[a]			
		DP Oligogalacturonide			
Time (h) Post-inoculation	Treatment[b]	10	11	12	13
4	*Xcm*	3.7 ± 0.0	2.3 ± 0.2	1.7 ± 0.6	1.7 ± 0.8
4	*Xcm*-BH	8.4 ± 0.4	5.5 ± 0.1	3.5 ± 0.0	3.3 ± 0.6
4	Mock-	20.2 ± 2.5	13.8 ± 1.1	9.6 ± 0.2	7.1 ± 0.5
8	*Xcm*	9.7 ± 3.5	5.1 ± 2.4	3.4 ± 1.2	1.2 ± 0.7
8	*Xcm*-BH	13.6 ± 3.3	7.6 ± 3.0	8.8 ± 3.0	3.1 ± 1.9
8	Mock-	9.2 ± 0.6	4.9 ± 0.0	3.3 ± 0.6	1.5 ± 0.8
8	Mock-BH	7.4	5.2	2.3	1.3
17	*Xcm*	6.5 ± 0.3	3.1 ± 0.1	2.7 ± 0.7	0.7 ± 0.3
17	*Xcm*-BH	9.5 ± 3.1	6.2 ± 1.7	6.3 ± 2.0	2.3 ± 0.2
17	Mock-	5.2 ± 1.2	2.9 ± 0.2	1.8 ± 0.4	0.7 ± 0.3

[a]Average of two replicates ± standard error. Mock-BH values were from one replicate. No values were reported for mock-BH at 4 h because of insufficient sample. Mock-BH sample at 17 h was lost during HW40S chromatography.

[b]BH denotes that sample has undergone saponification as described in the experimental section. Inoculum concentration for IWF collected at 4 h was 9.01 x 10^7 cfu mL^{-1}. Inoculum concentration for IWF collected at 8 h and 17 h post-inoculation was 1.02 x 10^8 cfu mL^{-1}.

TABLE 11

LEVELS OF PHYTOALEXIN-ELICITOR-ACTIVE OLIGOGALACTURONIDES FOUND IN IWF AT VARIOUS TIMES FROM *Xcm*- AND MOCK-INOCULATED FOURTEEN-DAY-OLD SUSCEPTIBLE WESTBURN (4.0) COTTON COTYLEDONS USING CDTA AS EXTRACTANT

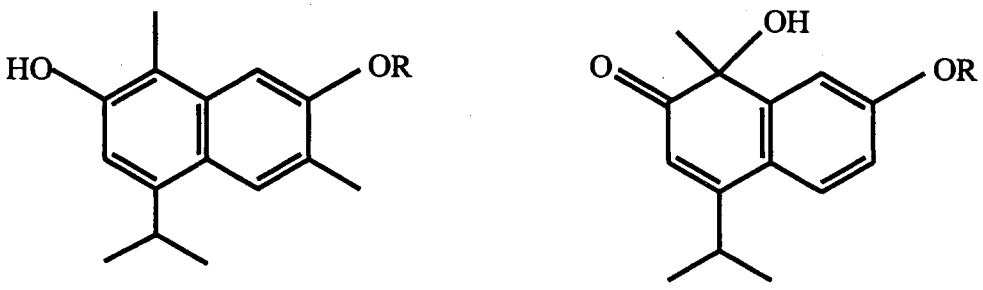
		Levels Found (pmol cm^{-2})[a]			
		DP Oligogalacturonide			
Time (h) Post-inoculation	Treatment[b]	10	11	12	13
4	*Xcm*	3.7	2.2	1.6	1.5
4	Mock-	5.0	3.0	3.0	2.7
8	*Xcm*	9.4 ± 1.3	5.9 ± 0.7	4.7 ± 0.4	1.7 ± 0.4
8	*Xcm*-BH	7.3 ± 0.6	4.2 ± 0.4	3.4 ± 0.3	1.2 ± 0.5
8	Mock-	8.2 ± 2.2	3.9 ± 1.4	2.7 ± 0.1	1.5 ± 0.6
8	Mock-BH	7.3	2.7	2.0	0.6
17	*Xcm*	13.4 ± 2.3	9.0 ± 1.0	8.8 ± 1.6	4.3 ± 1.0
17	*Xcm*-BH	9.2 ± 0.6	4.9 ± 0.6	4.0 ± 1.5	2.0 ± 0.4
17	Mock-	8.3 ± 0.7	3.7 ± 0.9	3.0 ± 0.4	1.4 ± 0.5

[a]Average of two replicates ± standard error. Values from 4 h post-inoculation were from one replicate; the second replicate could not be accurately quantitated because PL product dp=5 was used as internal standard as too low a level. Mock-BH values were from one replicate. No values were reported for *Xcm*-BH and mock-BH at 4 h and mock-BH at 17 h because of insufficient samples.

[b]BH denotes that sample has undergone saponification as described in the experimental section. Inoculum concentration for IWF collected at 4 h was 9.01×10^7 cfu mL^{-1}. Inoculum concentration for IWF collected at 8 h and 17 h post-inoculation was 1.02×10^8 cfu mL^{-1}.

APPENDIX B

FIGURES



1 R = H
2 R = CH_3

3 R = H
4 R = CH_3

Figure 1. Structures of 2,7-Dihydroxycadalene (1), 2-Hydroxy-7-methoxycadalene (2), Lacinilene C (3), and Lacinilene C 7-methyl Ether (4).

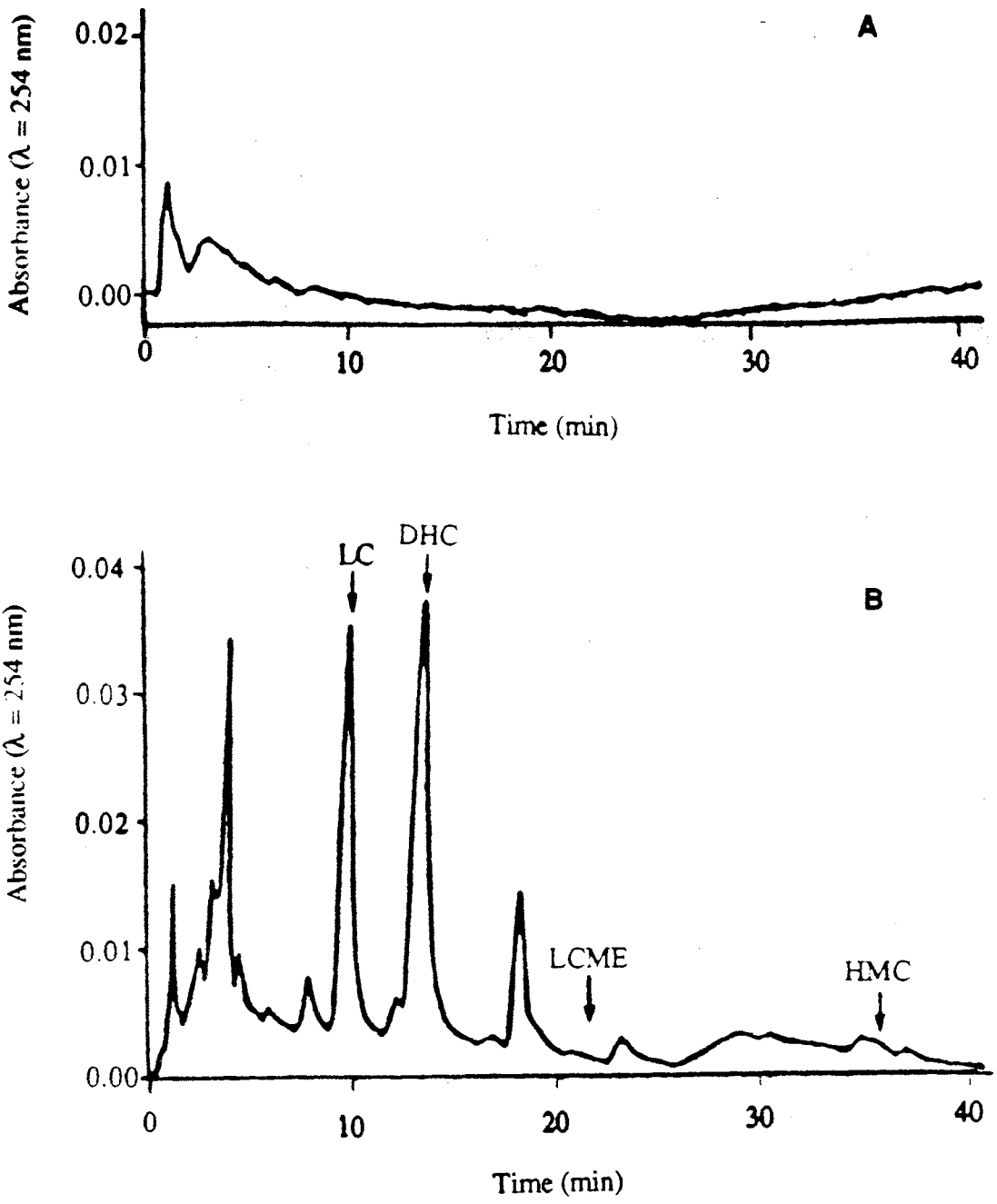


Figure 2. Phytoalexin Elicitation in Resistant OK 1.2 Cotton Cotyledons. HPLC chromatograms of phytoalexins from samples harvested at 42 h post-infiltration. Injections represent the phytoalexin levels from 1.62 cm^2 of plant tissue; A, OK 1.2 cotyledons infiltrated with water at pH 7.00; B, OK 1.2 cotyledons infiltrated with oligogalacturonide fraction 375 (0.45 mg mL^{-1}). The experiment was done once.

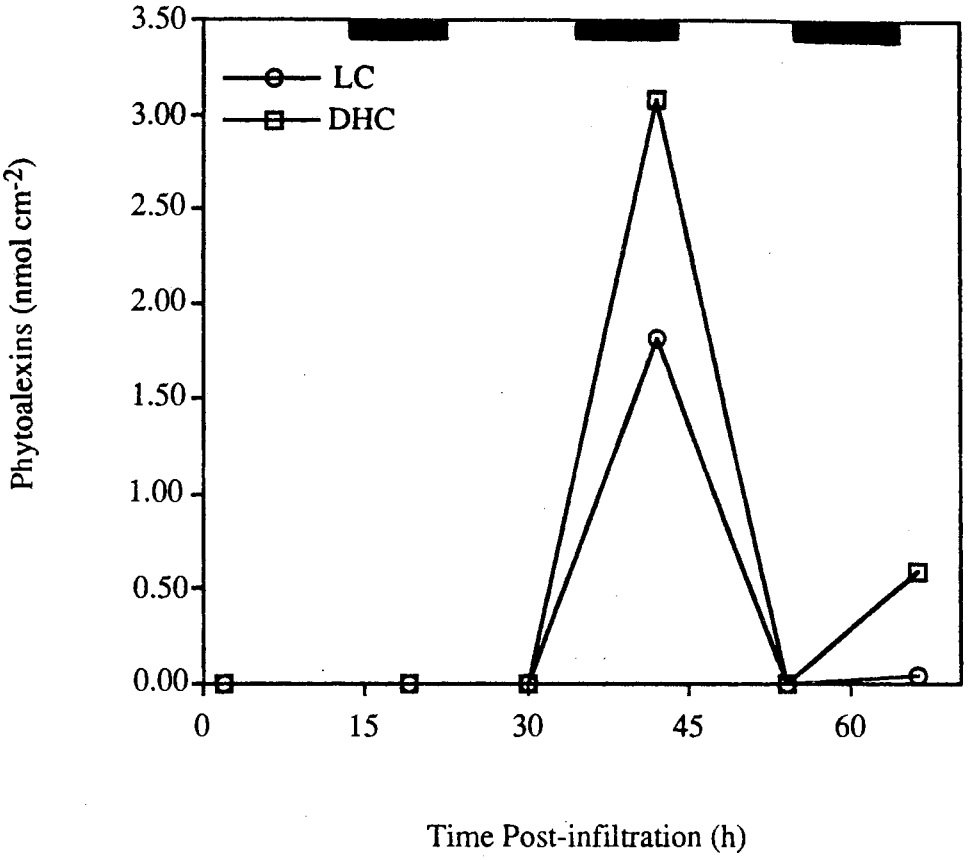


Figure 3. Levels of LC and DHC Elicited in Resistant OK 1.2 Cotyledons after Infiltration with a Mixture of Oligogalacturonides (dp = 8-16). Samples (4.00 cm^2) were harvested at different times after infiltration with oligogalacturonide fraction 375 (0.45 mg mL^{-1}) and were collected from 10 different cotyledons, pooled, and analyzed by HPLC. White and black bars on the x-axis denote periods of light and darkness, respectively, in the growth chamber.

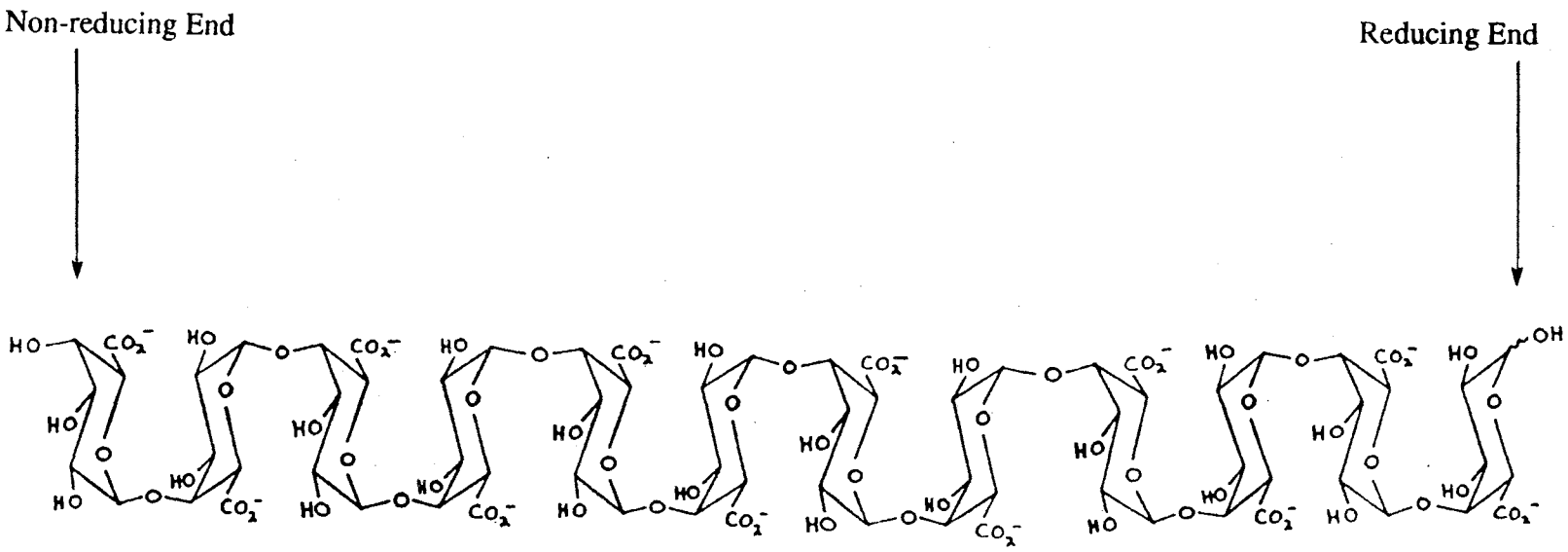


Figure 4. Structure of α-1,4-Dodecagalacturonide.

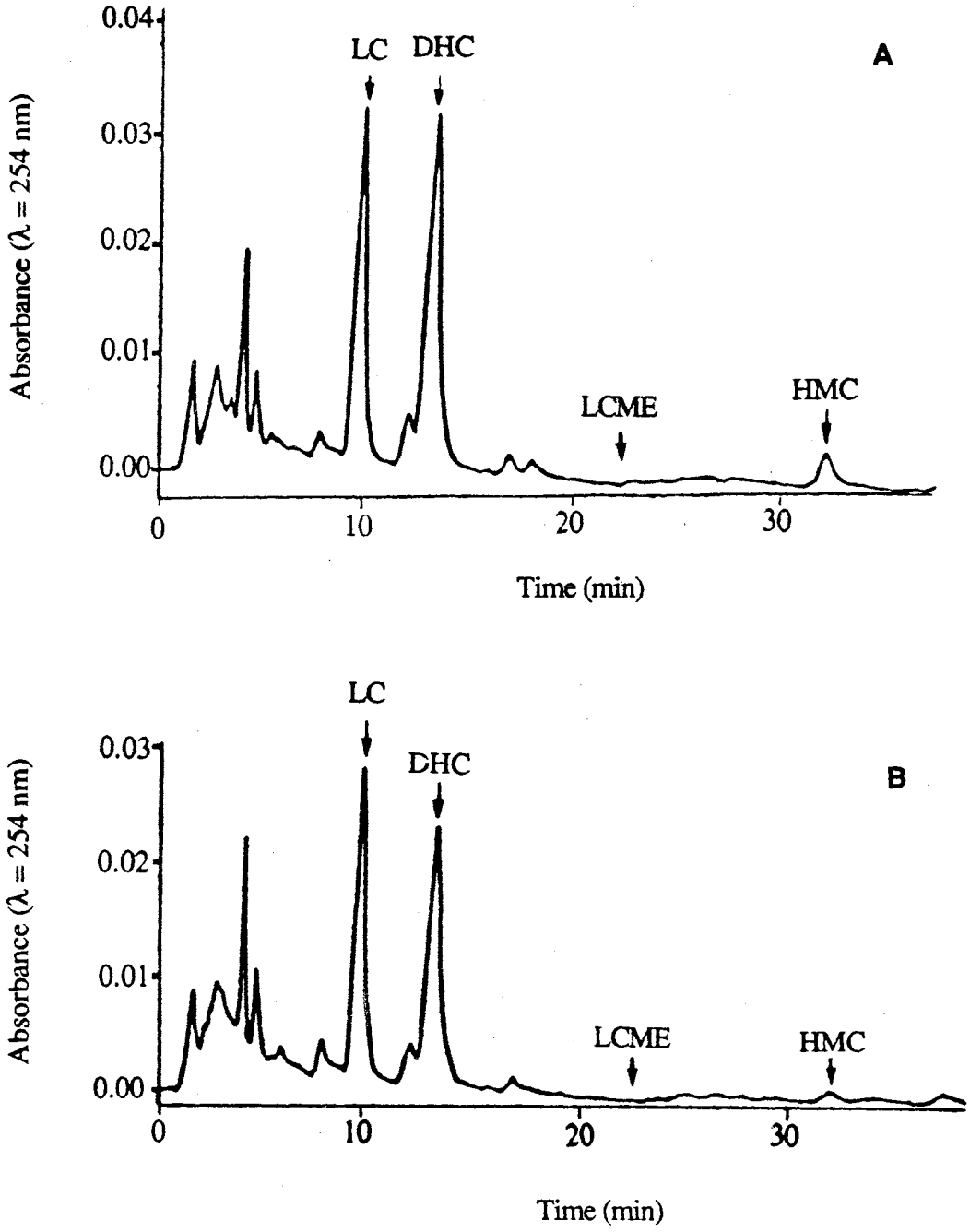


Figure 5. Stress Metabolite Elicitation in Resistant OK 1.2 Cotton Cotyledons after Infiltration with the Dodecagalacturonide. Sample HPLC chromatograms of stress metabolites from 13-day-old OK 1.2 cotton cotyledons infiltrated with the dodecagalacturonide. Injections represent 2.93 cm^2 plant tissue. Samples were harvested 40 h after infiltration; A, 0.068 mg mL^{-1}; and B, 0.137 mg mL^{-1}.

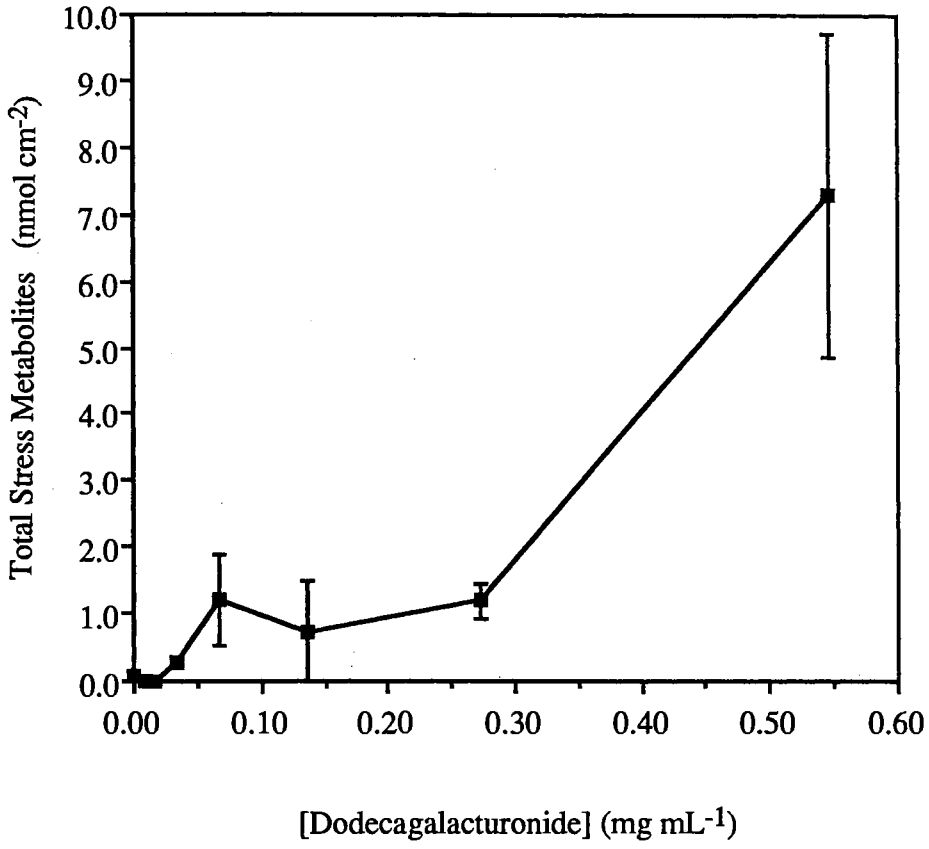


Figure 6. Total Stress Metabolites Elicited in OK 1.2 Cotton Cotyledons after Infiltration with Several Concentrations of the Dodecagalacturonide. Samples (6.0 cm^2) were harvested 40 h after infiltration. Error bars represent standard error based on three replicates. Each value represents the sum of LC, DHC, and HMC. The experiment was done once.

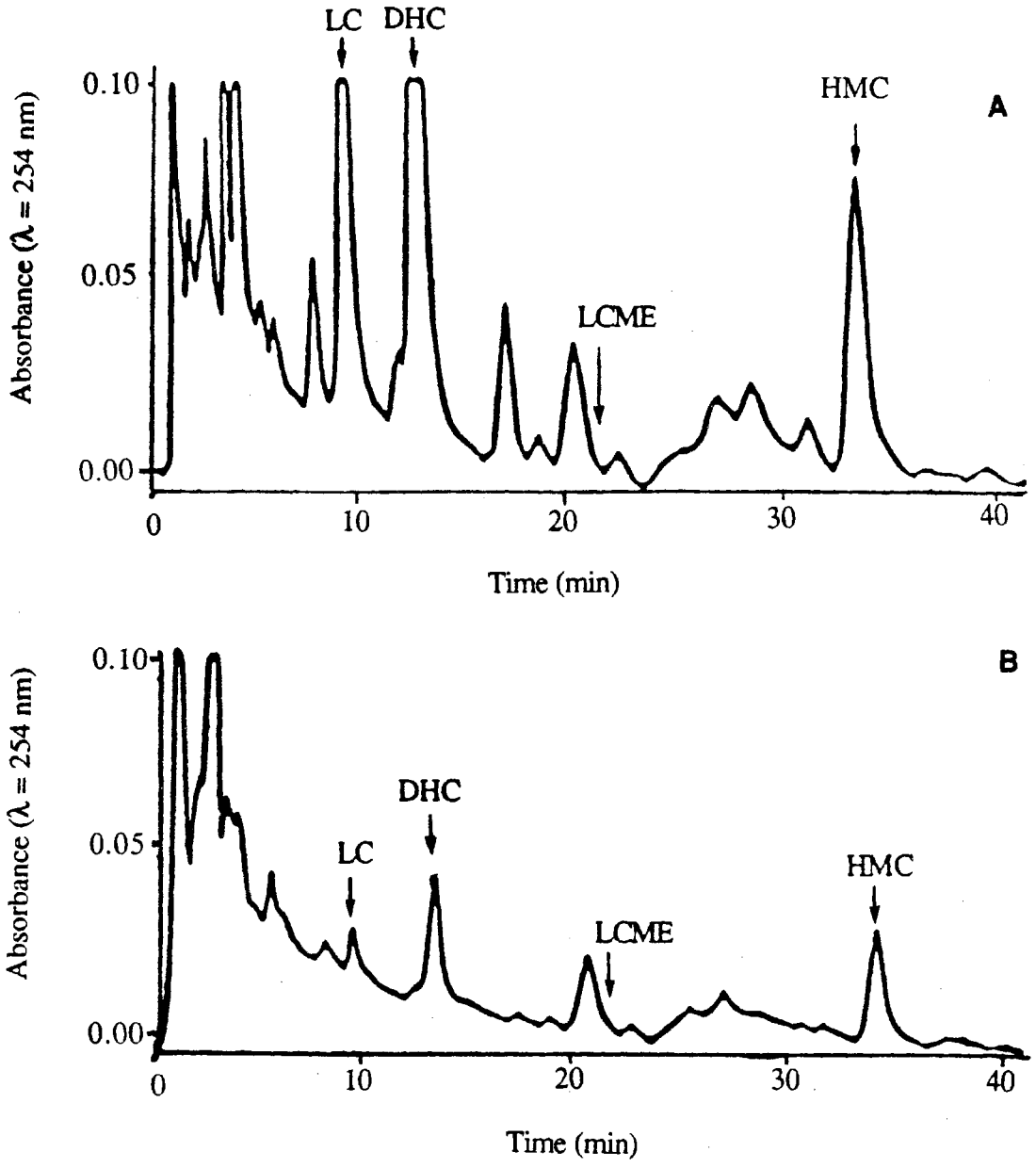


Figure 7. Stress Metabolite Elicitation in Resistant OK 1.2 and Susceptible Ac 44E after Infiltration with the Tridecagalacturonide. Sample HPLC chromatograms of stress metabolites from 13-day-old cotton cotyledons infiltrated with increasing concentration of the tridecagalacturonide. Samples were harvested 40 h after infiltration; A, OK 1.2 plus 0.50 mg mL^{-1}; and B, Ac 44E plus 0.50 mg mL^{-1} tridecagalacturonide.

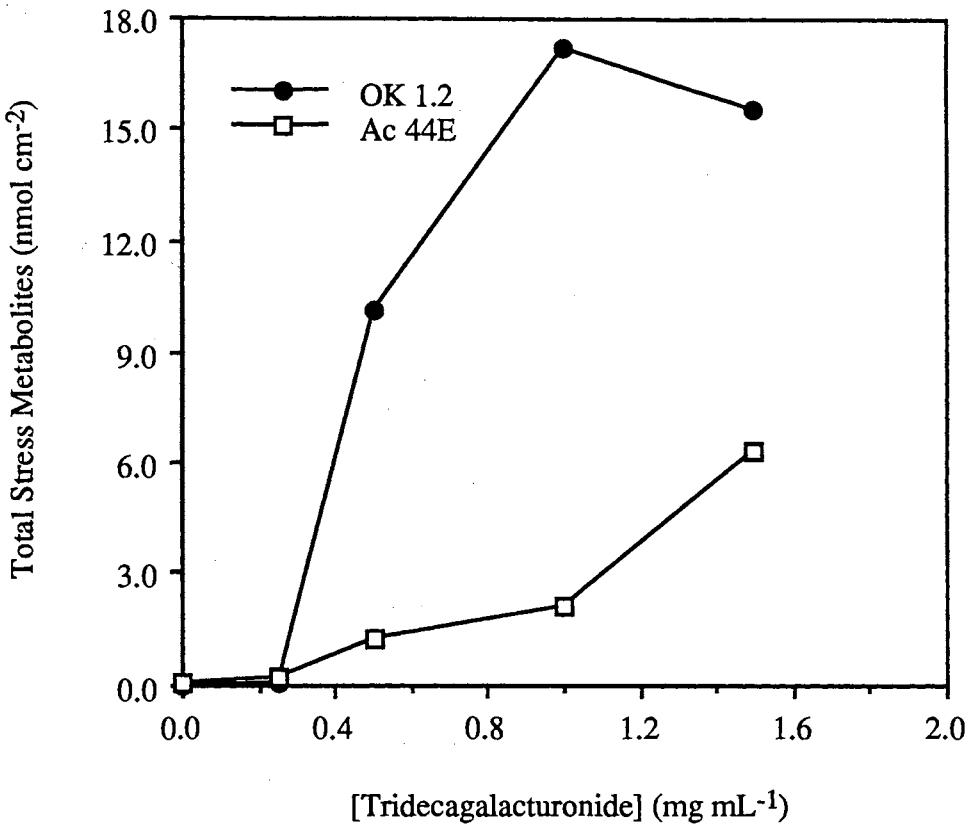


Figure 8. Total Stress Metabolites Elicited in Resistant (OK 1.2) and Susceptible (Ac 44E) Cotton Cotyledons after Infiltration with Several Concentrations of the Tridecagalacturonide. Samples (1.80 cm^2 for OK 1.2 and 3.00 cm^2 for Ac 44E) were harvested 40 h after infiltration. Each value represents the sum of LC, DHC, and HMC. The experiment was done once.

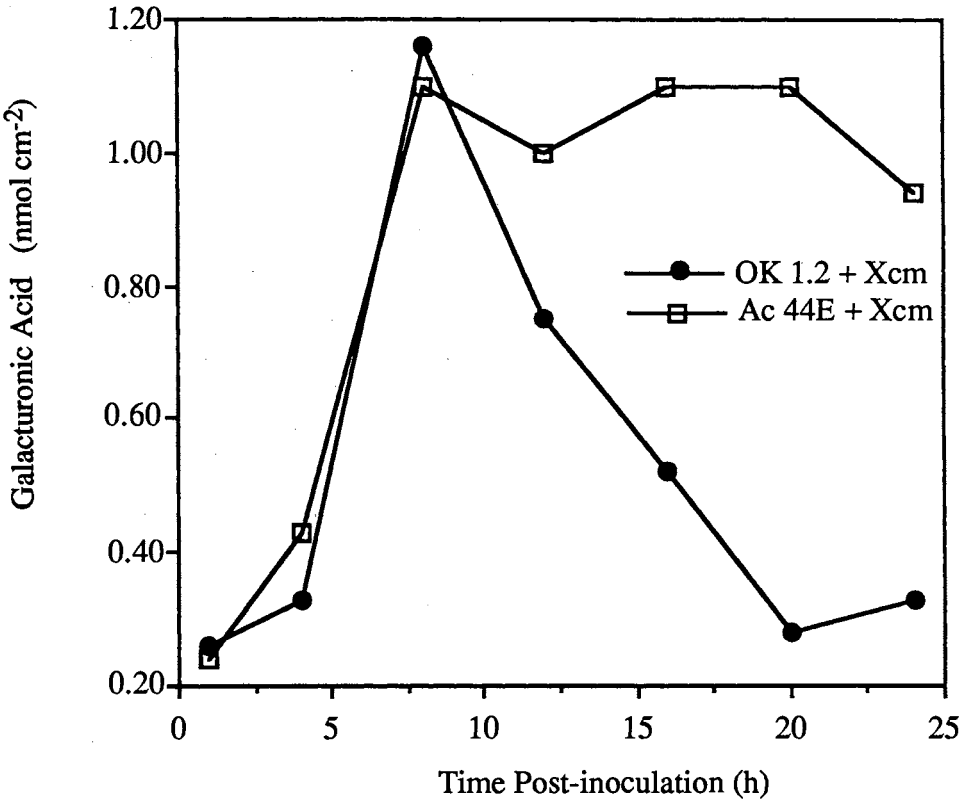
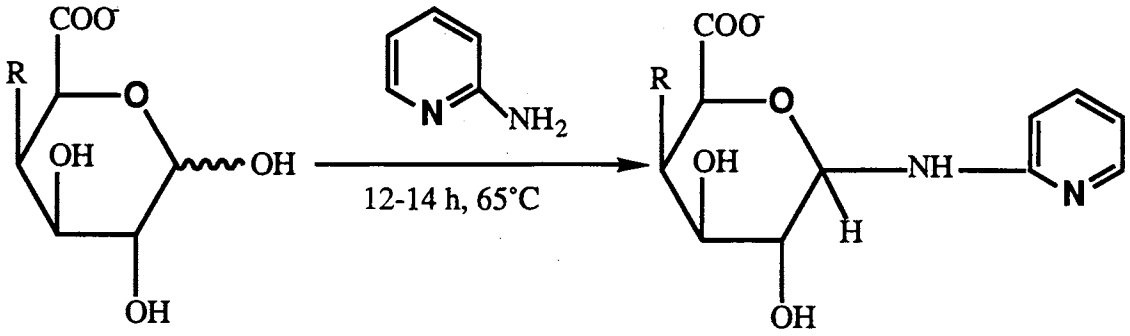


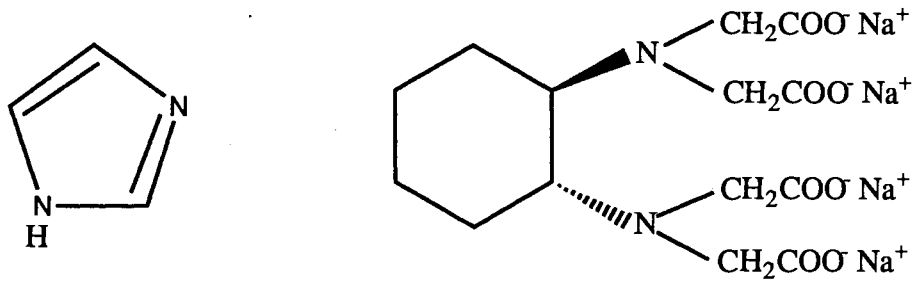
Figure 9. Galacturonic Acid Levels in IWF Prepared at Various Times from 14-day-old Resistant OK 1.2 Inoculated with *Xcm* (1.71 x 10^8 cfu mL^{-1}) and Susceptible Ac 44E Cotyledons Inoculated with *Xcm* (1.66 x 10^8 cfu mL^{-1}). Each value represents the average galacturonic acid level from two replicates. The experiment was done once.



Oligogalacturonide with Reducing End

N-(2-Pyridinyl)-glycosylamine

Figure 10. Reaction Showing the Condensation of the Reducing End of an Oligogalacturonide with 2-Aminopyridine.



1 2

Figure 11. Structures of Imidazole (1), and the Sodium Salt of *trans*-1,2-Diaminocyclohexane-*N,N,N',N'*-tetraacetic Acid (CDTA) (2).

Figure 12. Chromatographic Summary of the Recovery Studies with the Undecagalacturonide: Representative Fractionation of IWF on the Semi-preparative HW40S Gel Permeation Column. WbM (0.0) cotton cotyledons were infiltrated with 0.5 mg mL^{-1} undecagalacturonide-rich fraction and IWF prepared immediately after the water of infiltration had transpired using water, imidazole, or CDTA as extracting solvent. The undecagalacturonide-infiltrated cotyledons were extracted twice with the various extractants. Fractions underlined were pooled for further analysis; A, Amount of standard undecagalacturonide (67.5 μg) added to 15 cm^2 of plant tissue based on the intercellular volume of 9 μL cm^{-2}; B, IWF from cotton cotyledons treated with the undecagalacturonide and extracted with CDTA (extract 1); and C, IWF from cotton cotyledons infiltrated with water (control) and extracted with CDTA (extract 1).

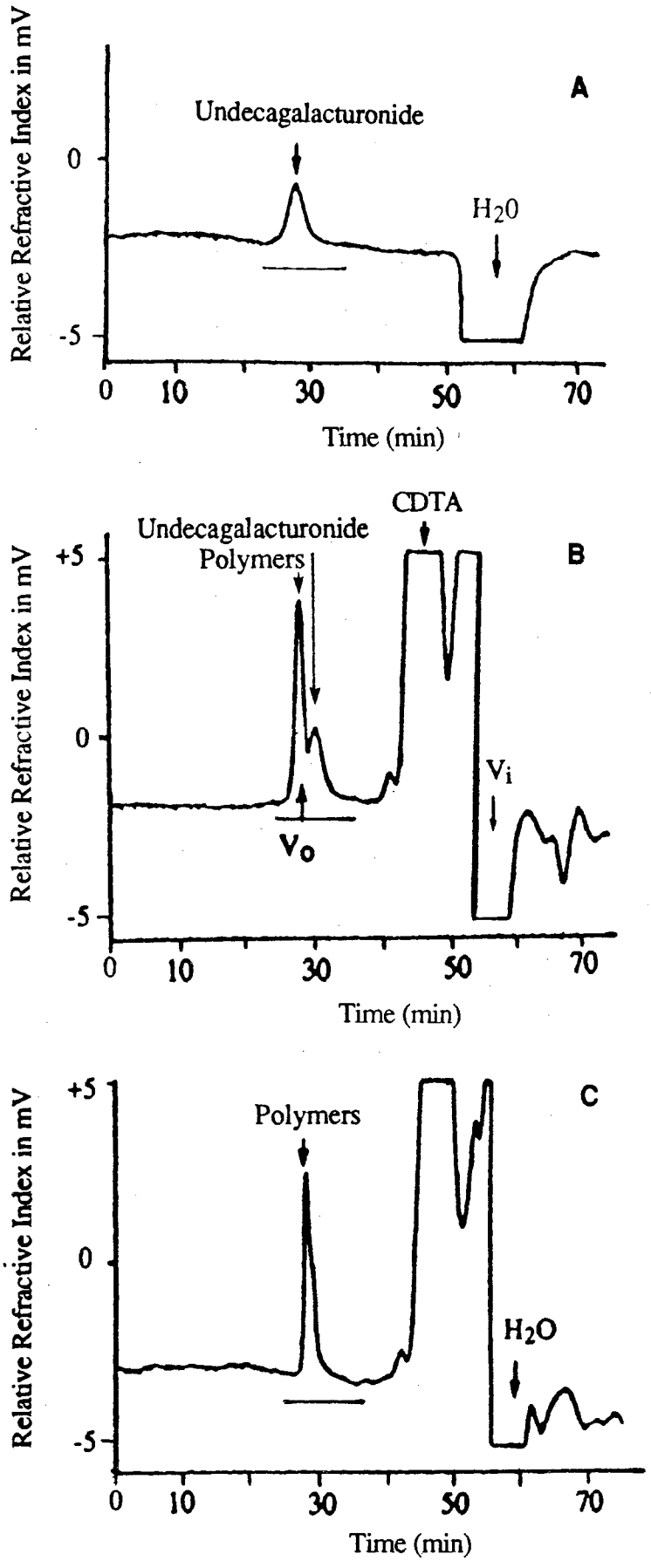
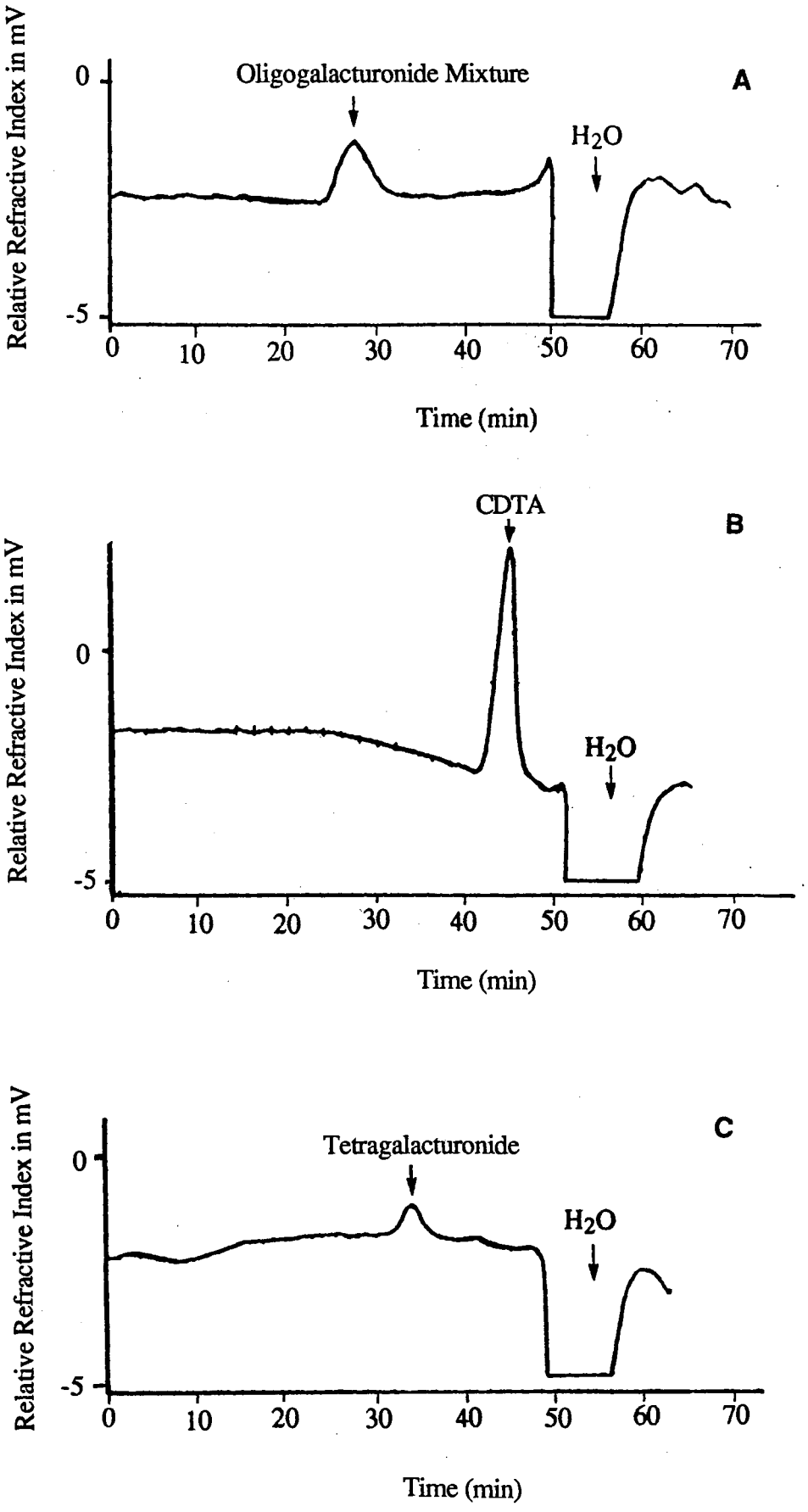
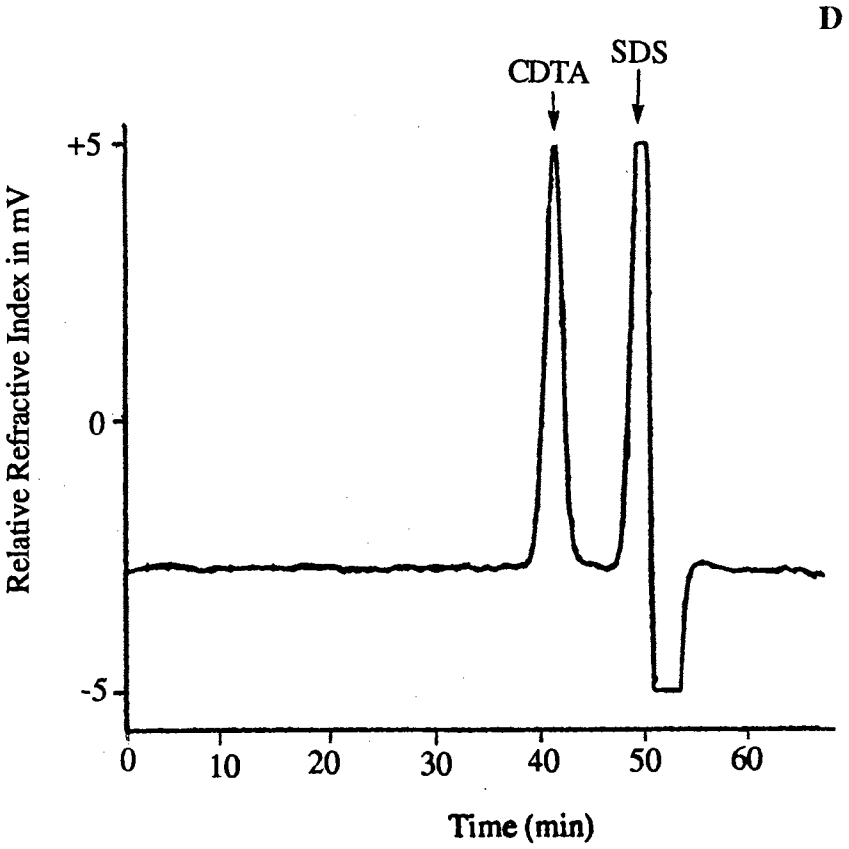

A
Undecagalacturonide
H_2O
Relative Refractive Index in mV
0
-5
0
10
30
50
70
Time (min)
B
CDTA
Undecagalacturonide
Polymers
+5
0
-5
V_i
V_o
Relative Refractive Index in mV
0
10
30
50
70
Time (min)
C
Polymers
+5
0
-5
H_2O
Relative Refractive Index in mV
0
10
30
50
70
Time (min)

Figure 13. Typical Elution Times of Molecules of Interest on the Semi-preparative HW40S Gel Permeation Column. A, Mixture of oligogalacturonides (*i.e.*, fraction 375, dp = 8-16); B, CDTA; C, Tetragalacturonide; D, CDTA and SDS; and E, Dodecagalacturonide.


Relative Refractive Index in mV
0
-5
Oligogalacturonide Mixture
A
H_2O
0
10
20
30
40
50
60
70
Time (min)
CDTA
B
H_2O
Time (min)
Tetragalacturonide
C
H_2O
Time (min)


D
CDTA
SDS
+5
0
-5
Relative Refractive Index in mV
0
10
20
30
40
50
60
Time (min)

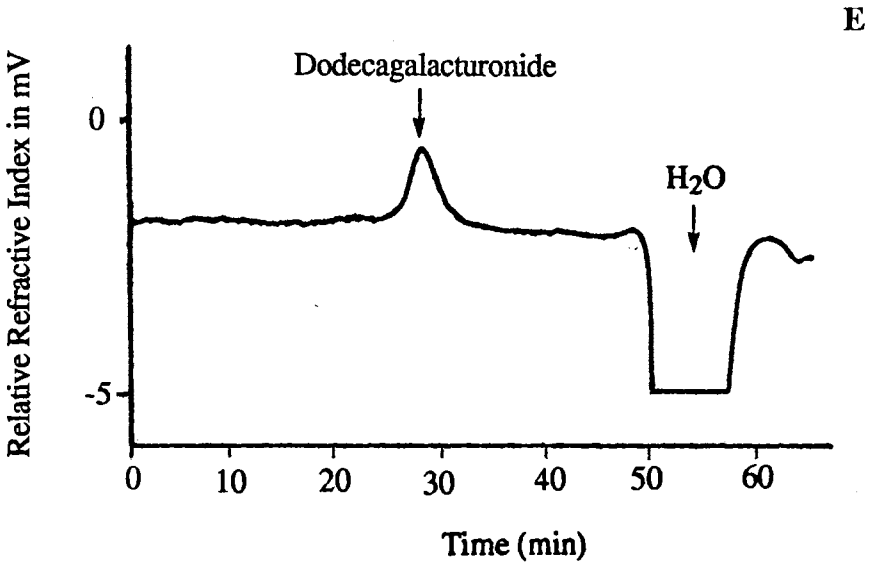

E
Dodecagalacturonide
H2O
0
-5
Relative Refractive Index in mV
0
10
20
30
40
50
60
Time (min)

Figure 14. Typical Elution Times of Molecules of Interest on the Preparative HW40S Gel Permeation Column; A, Citrus pectin and CDTA; B, Mixture of oligogalacturonides (*i.e.*, fraction 375, dp = 8-16) and CDTA; and C, 4,5-Unsaturated PL product (dp = 5).

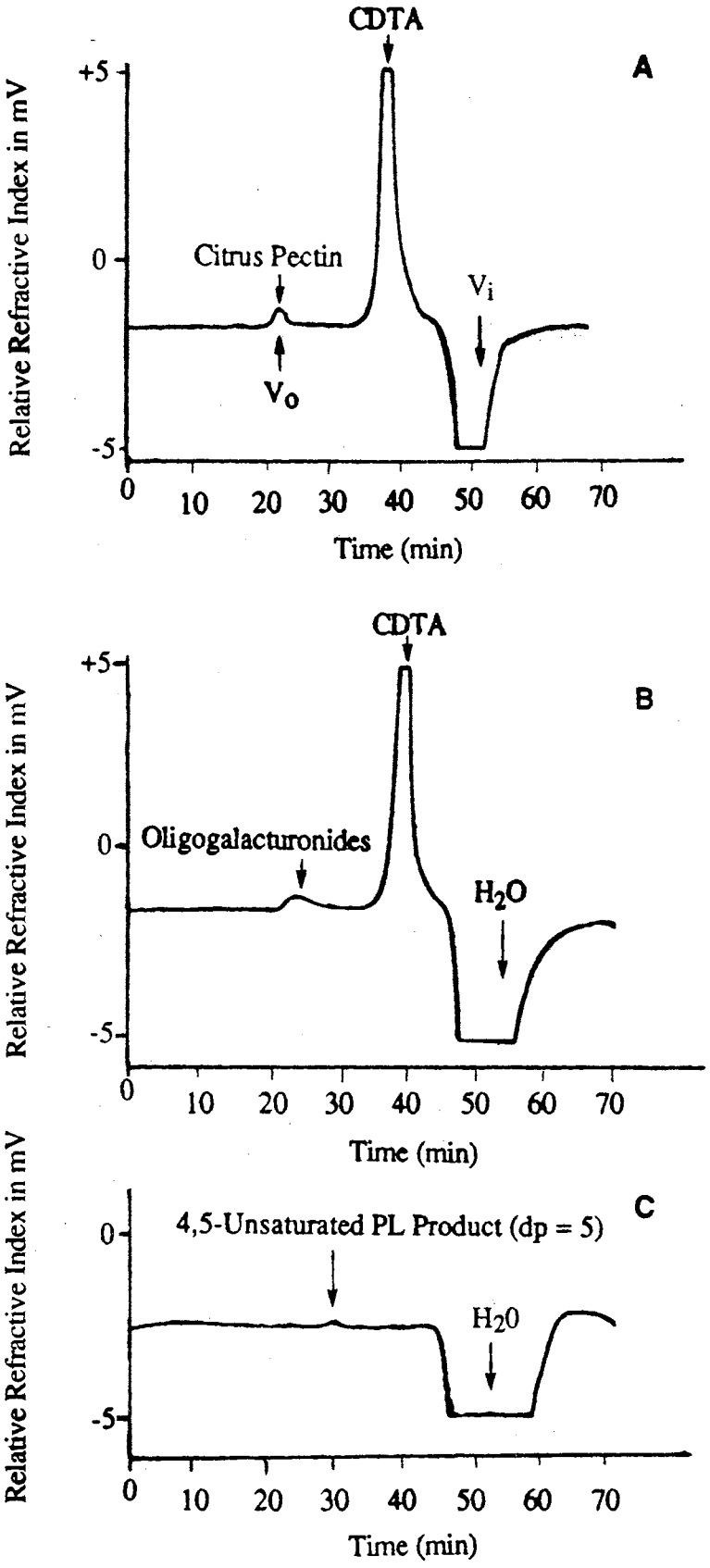

CDTA
A
+5
0
-5
Relative Refractive Index in mV
Citrus Pectin
V_i
V_o
0 10 20 30 40 50 60 70
Time (min)
CDTA
B
+5
0
-5
Relative Refractive Index in mV
Oligogalacturonides
H_2O
0 10 20 30 40 50 60 70
Time (min)
C
0
-5
Relative Refractive Index in mV
4,5-Unsaturated PL Product (dp = 5)
H_20
0 10 20 30 40 50 60 70
Time (min)

Figure 15. Chromatographic Summary of the Recovery Studies with the Undecagalacturonide: Representative Fractionation of IWF on the TSK2000SW Gel Permeation Column. Fractions co-chromatographing with and close to the void volume, V_o, during HW40S fractionation were collected and purified further on TSK2000SW column. Fractions underlined were pooled for further analysis; A, Standard undecagalacturonide-rich fraction recovered from the HW40S fractionation; B, Cotton cotyledons infiltrated with the undecagalacturonide-rich fraction, extracted with CDTA (first extract), and purified in HW40S column; and C, Cotton cotyledons infiltrated with the undecagalacturonide-rich fraction, extracted with CDTA (second extract), and purified in HW40S column.

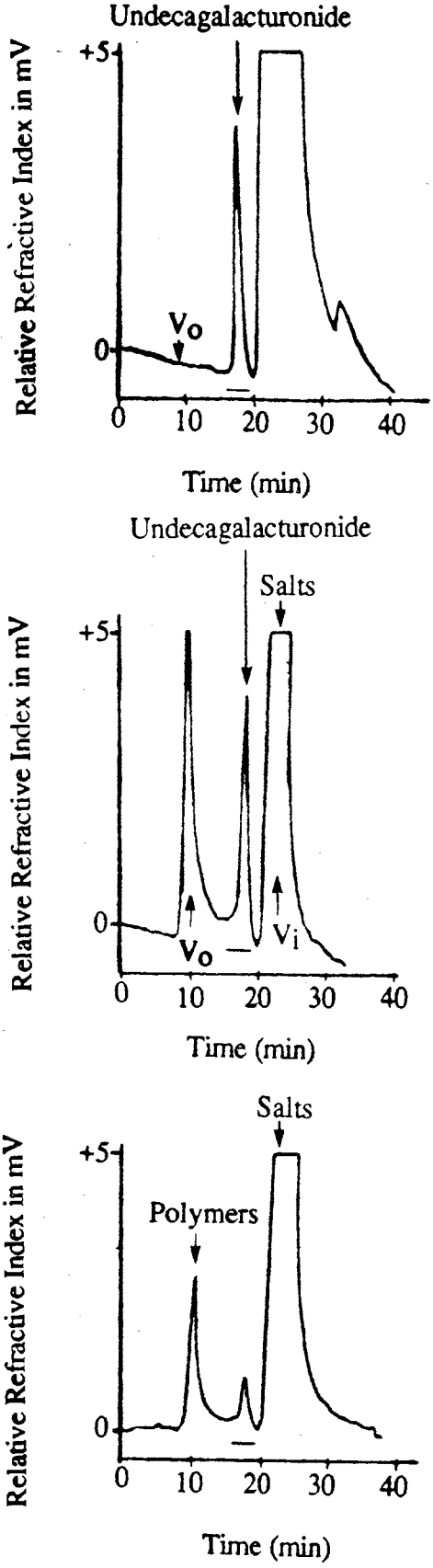

Undecagalacturonide
A
+5
0
Relative Refractive Index in mV
Vo
0
10
20
30
40
Time (min)
Undecagalacturonide
Salts
B
+5
0
Relative Refractive Index in mV
Vo
Vi
0
10
20
30
40
Time (min)
Salts
C
+5
Polymers
0
Relative Refractive Index in mV
0
10
20
30
40
Time (min)

Figure 16. Typical Elution Times of Molecules of Interest on the TSK2000SW Gel Permeation Column; A, Citrus pectin and the mixture of oligogalacturonides (*i.e.*, fraction 375, dp = 8-16); B, Mixture of oligogalacturonides; and C, CDTA.

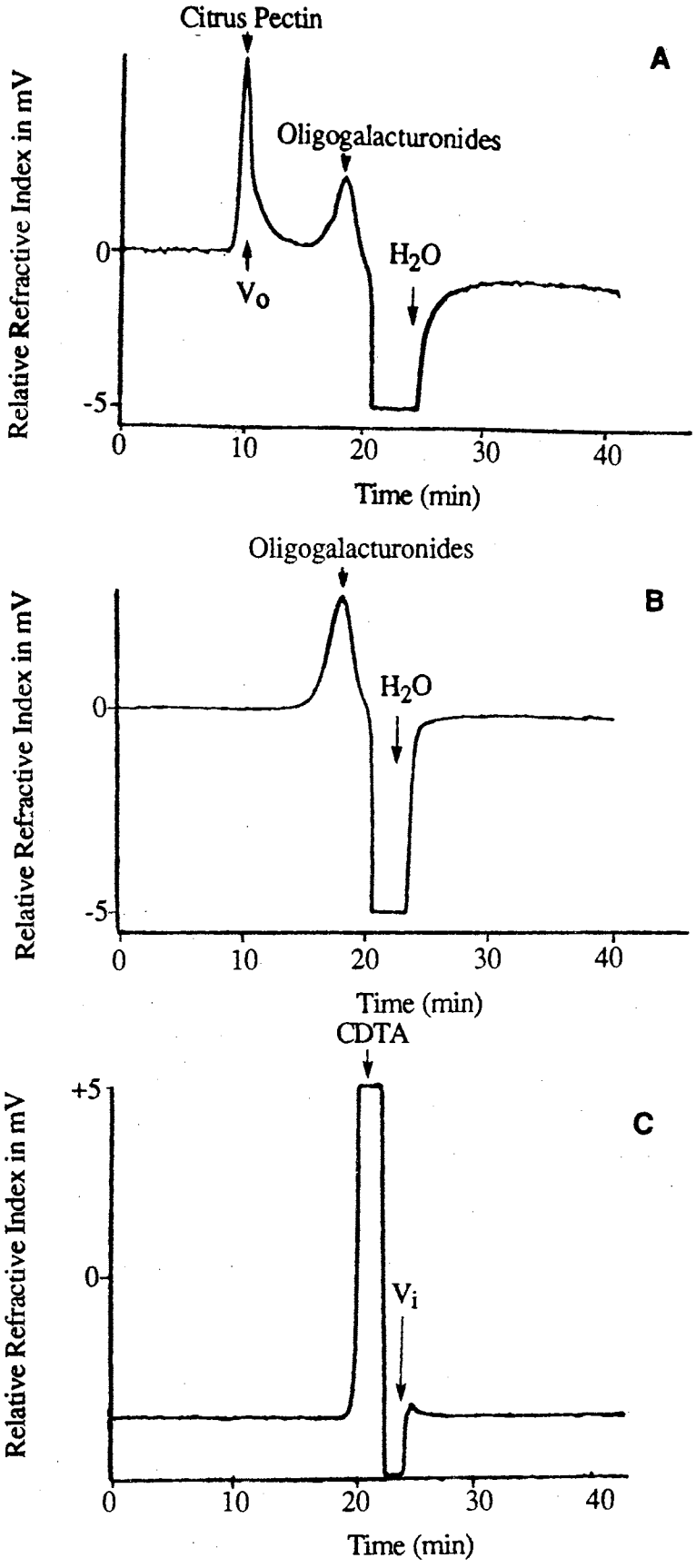

Citrus Pectin
A
Oligogalacturonides
H_2O
V_o
Relative Refractive Index in mV
0
-5
0
10
20
30
40
Time (min)
Oligogalacturonides
B
H_2O
Relative Refractive Index in mV
0
-5
0
10
20
30
40
Time (min)
CDTA
C
+5
0
V_i
Relative Refractive Index in mV
0
10
20
30
40
Time (min)

Figure 17. Demonstration of the Presence of Oligogalacturonides and the Pectic Nature of the Molecules Producing the Peaks in the IWF Derived from Resistant WbM (0.0) Inoculated with *Xcm* (9.00×10^7 cfu mL^{-1}): Treatment with PG from *Erwinia carotovora*; A, IWF at 8 h post-inoculation from WbM (0.0) cotyledons inoculated with *Xcm*; and B, The same IWF as in A plus 0.10 unit PG. Chromatography was performed on PA-1 anion-exchange column using the sodium acetate gradient described in the experimental section.

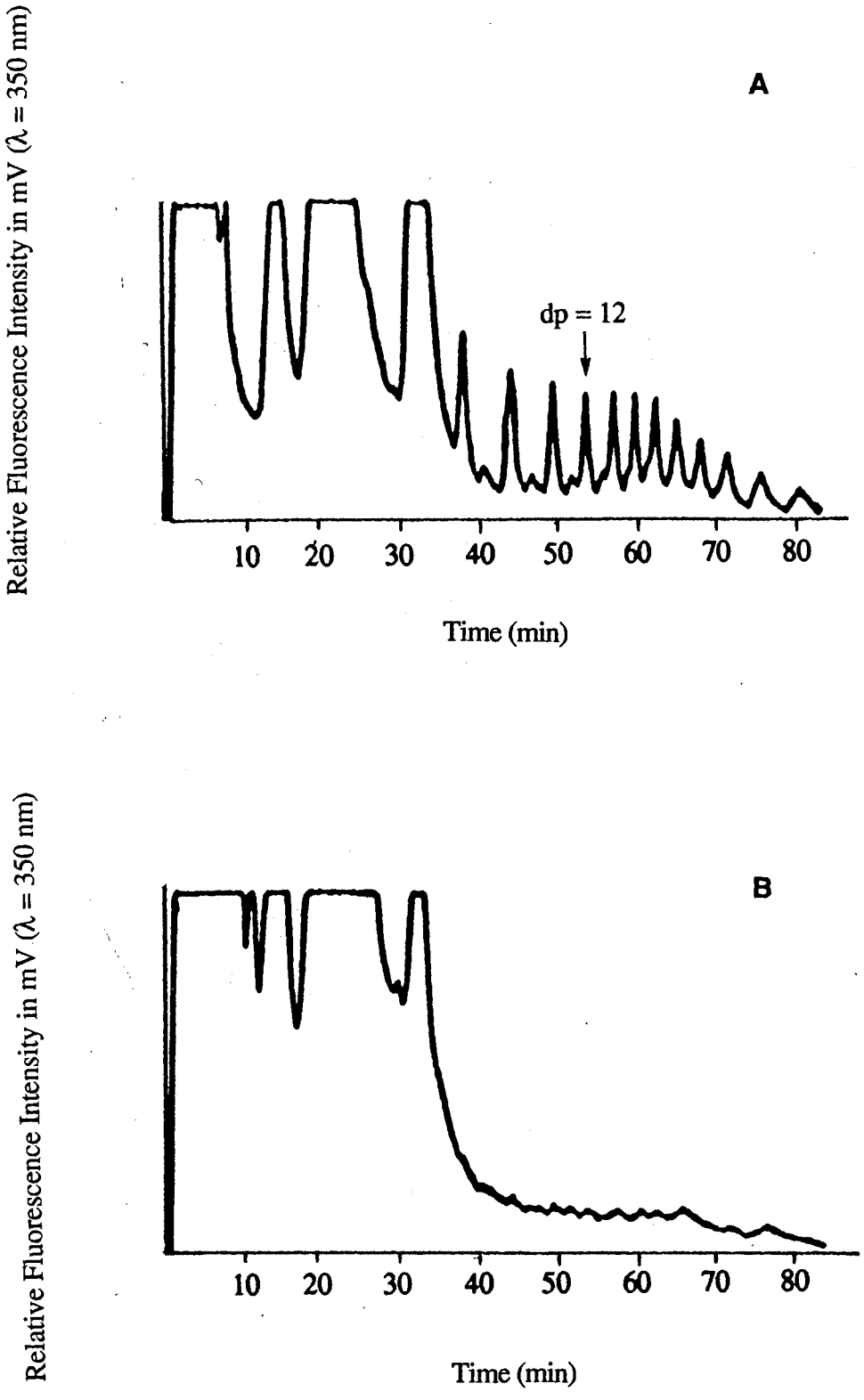
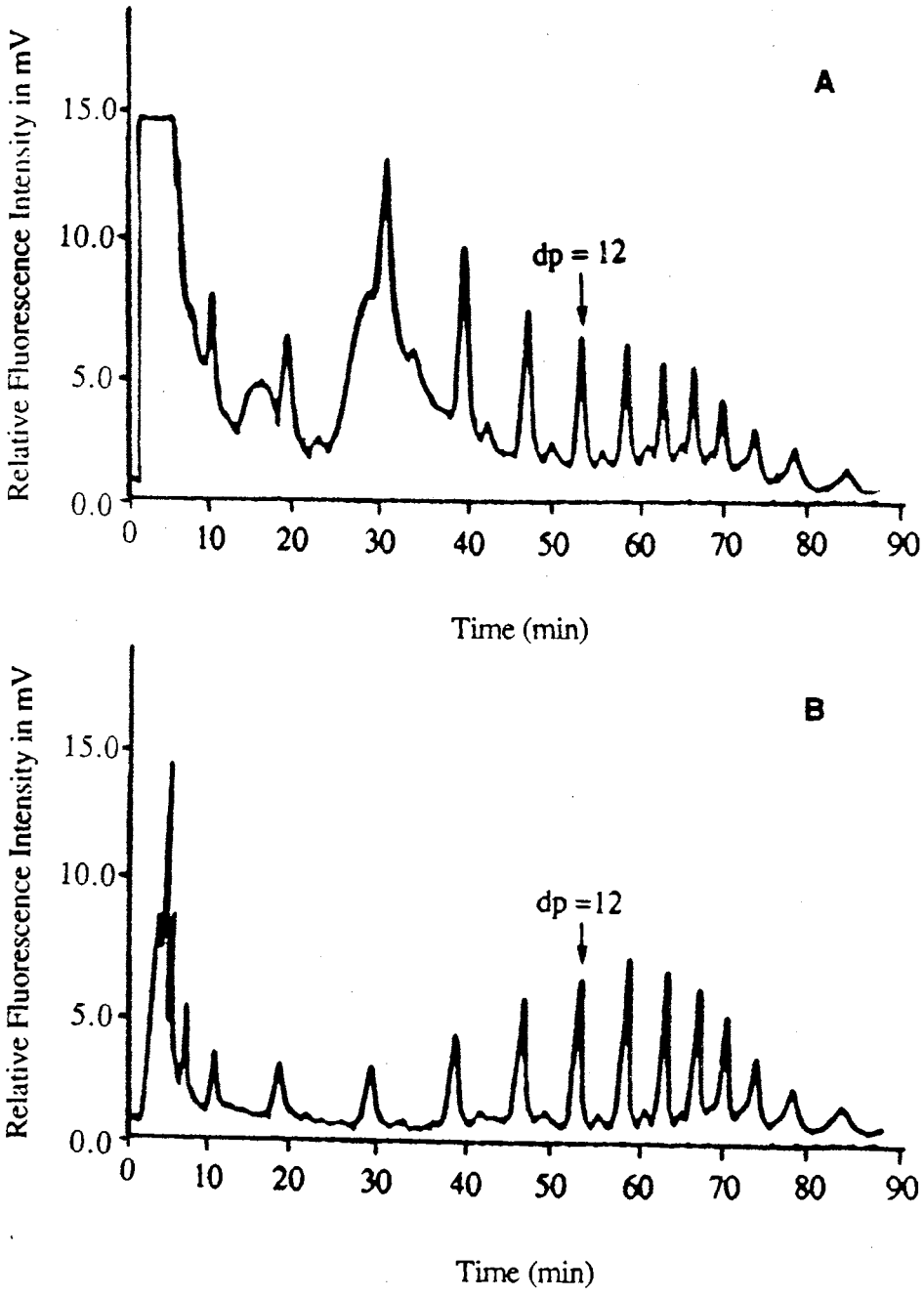

A
Relative Fluorescence Intensity in mV (λ = 350 nm)
dp = 12
10
20
30
40
50
60
70
80
Time (min)
B
Relative Fluorescence Intensity in mV (λ = 350 nm)
10
20
30
40
50
60
70
80
Time (min)

Figure 18. Demonstration of the Pectic Nature of IWF from Resistant WbM (0.0) Cotyledons Inoculated with *Xcm*: Comparison of Retention Times with Standard Oligogalacturonides Derived from Autoclave-hydrolysis of Polygalacturonic Acid; A, IWF obtained at 8 h post-inoculation from WbM (0.0) inoculated with *Xcm* (2.0×10^8 cfu mL^{-1}); and B, Standard mixture of oligogalacturonides obtained by autoclave-hydrolysis of polygalacturonic acid. Chromatography was performed using the sodium acetate gradient described in the experimental section.


A
Relative Fluorescence Intensity in mV
15.0
10.0
5.0
0.0
dp = 12
0
10
20
30
40
50
60
70
80
90
Time (min)
B
Relative Fluorescence Intensity in mV
15.0
10.0
5.0
0.0
dp =12
0
10
20
30
40
50
60
70
80
90
Time (min)

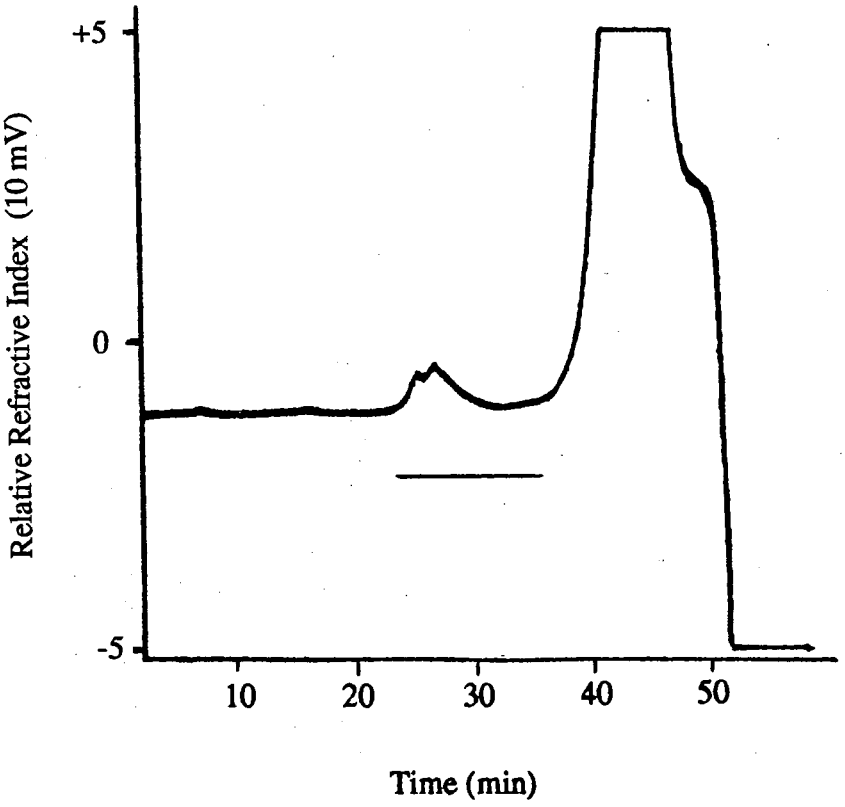
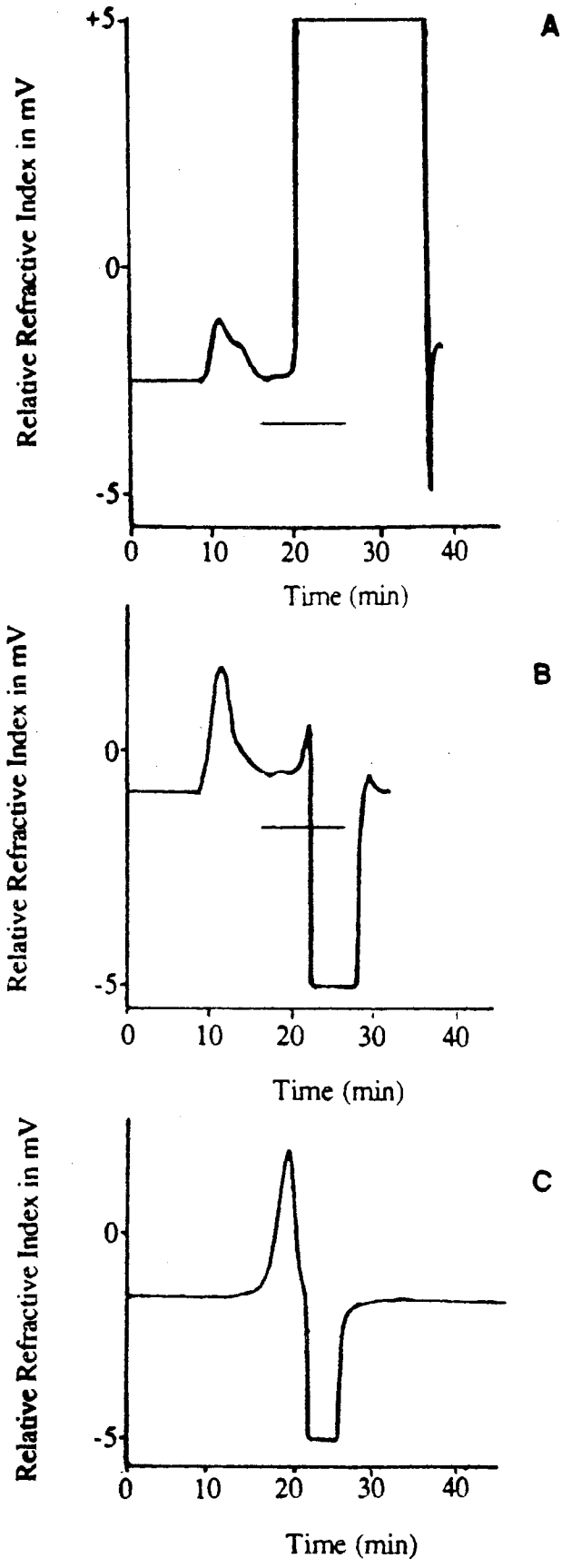


Figure 19. Gel Permeation Chromatography on the Preparative HW40S Column of IWF Obtained at 17 h Post-inoculation from 14-day-old WbM (0.0) Cotyledons Inoculated with *Xcm* (1.02×10^8 cfu mL^{-1}). The volume injected represents IWF equivalent to 50 cm^2 plant tissue plus internal standard. The fraction chromatographing between 22-36 ± 1 min (underlined), which includes the high-mol wt materials plus the oligogalacturonides and the internal standard, was collected in a silanized 50-mL Erlenmeyer flask as described in the experimental section.

Figure 20. Gel Permeation Chromatography on TSK2000SW of IWF Obtained from 14-day-old WbM (0.0) Cotyledons Inoculated with *Xcm*. IWF were chromatographed first on the HW40S preparative column to remove the extractant, CDTA, before chromatography on TSK2000SW; A, IWF (equivalent to 50 cm^2 plant tissue) obtained at 4 h post-inoculation from WbM (0.0) inoculated with *Xcm* (9.01 x 10^7 cfu mL^{-1}); B, IWF (equivalent to 50 cm^2 plant tissue) obtained at 8 h post-inoculation from WbM (0.0) inoculated with *Xcm* (1.02 x 10^8 cfu mL^{-1}); and C, Standard mixture of oligogalacturonides derived from autoclave-hydrolysis of polygalacturonic acid. The fraction chromatographing between 14-24 min (underlined), which include the oligogalacturonides and the internal standard, was pooled and dried.


A
+5
0
-5
Relative Refractive Index in mV
0
10
20
30
40
Time (min)
B
Relative Refractive Index in mV
0
-5
0
10
20
30
40
Time (min)
C
Relative Refractive Index in mV
0
-5
0
10
20
30
40
Time (min)

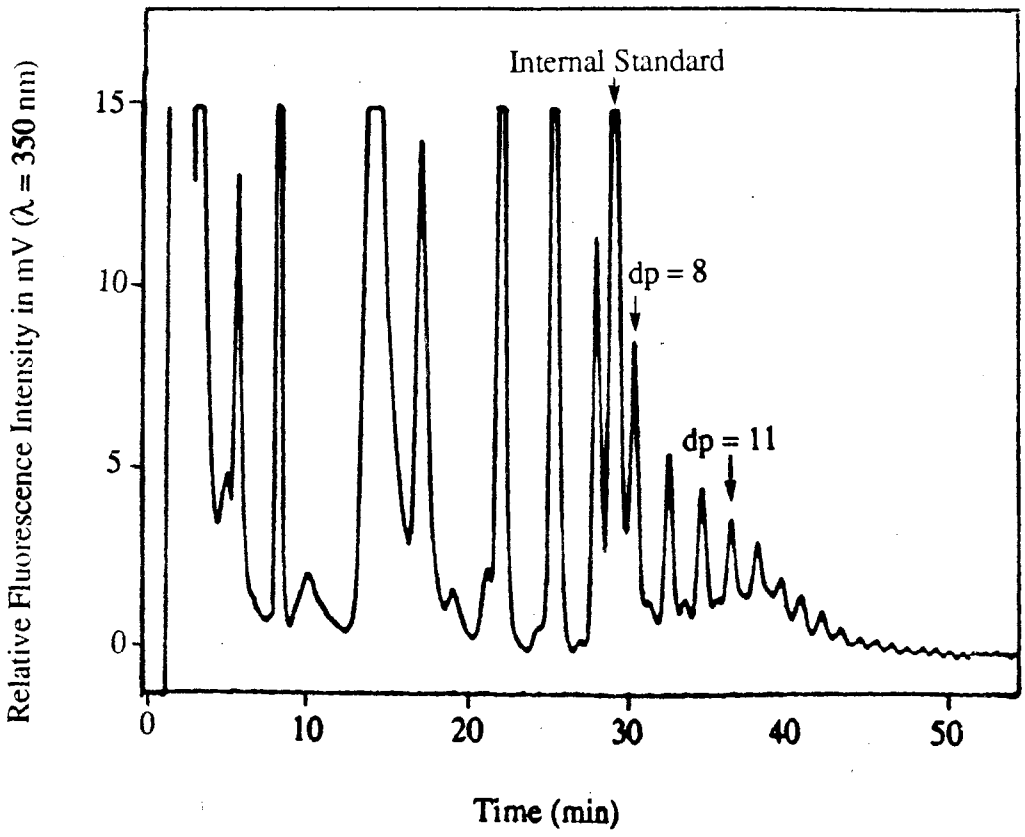


Figure 21. PA-1 Anion-exchange Chromatogram of IWF from WbM (4.0) Inoculated with *Xcm* (1.02 x 10^8 cfu mL^{-1}) at 8 h Post-inoculation. Injection represents 25 cm^2 of cotyledon. Sample was run using potassium oxalate buffered at pH 7.80 as described in the experimental section.

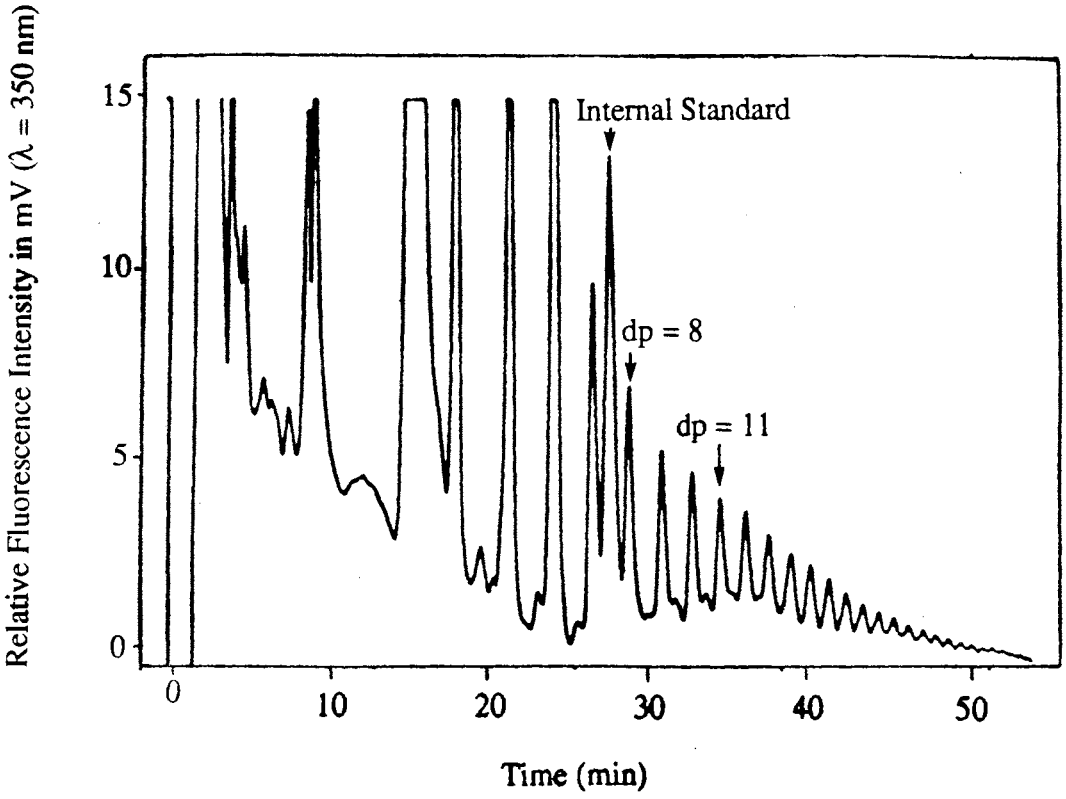


Figure 22. PA-1 Anion-exchange Chromatogram of IWF at 4 h Post-infiltration Collected from WbM (0.0) Infiltrated with Water Saturated with Calcium Carbonate (Bacterial Suspension Medium). Injection represents 25 cm^2 of cotyledon. Sample was run using potassium oxalate buffered at pH 7.80 as described in the experimental section.

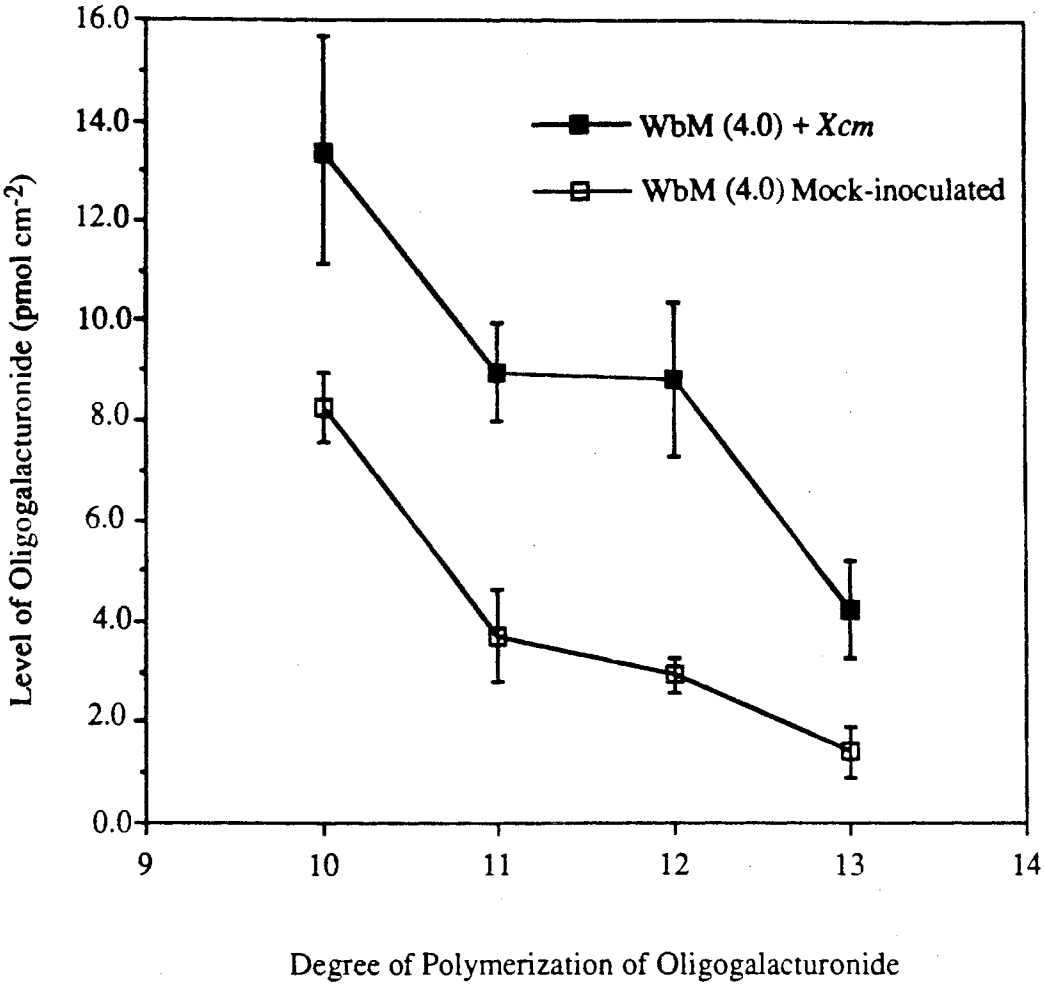


Figure 23. Levels of Elicitor-active Oligogalacturonides Found in the *Xcm*-inoculated and Mock-inoculated when IWF were Collected at 17 h Post-inoculation. Values plotted are the average of two replicates ± standard error. The experiment was done once.

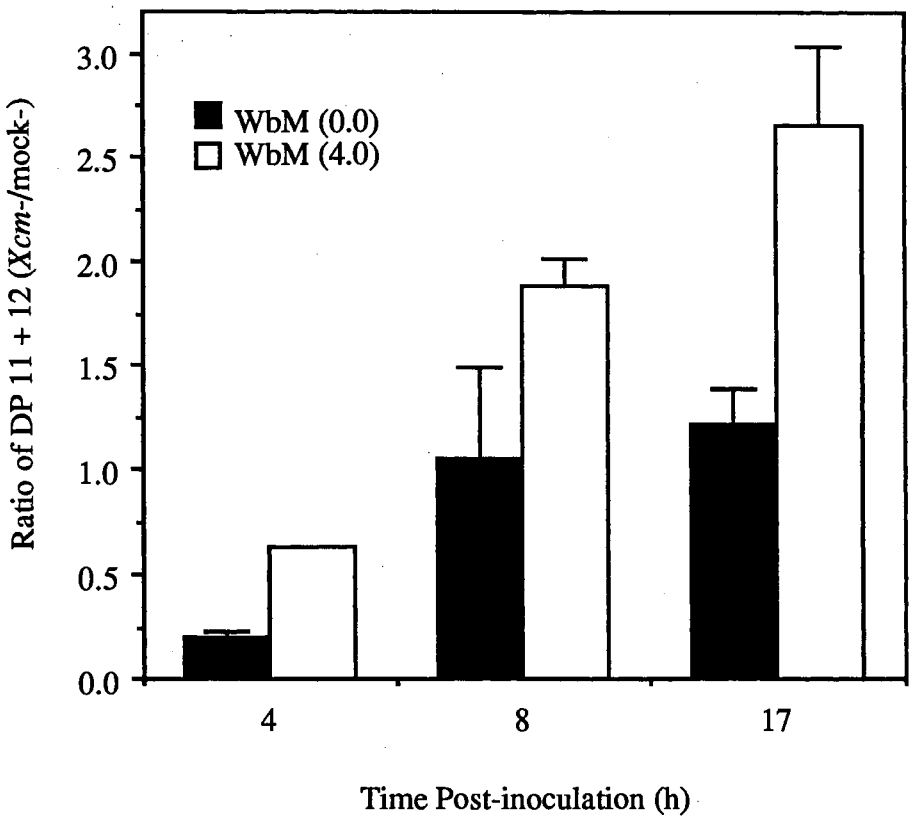


Figure 24. Relative Levels of Elicitor-active Oligogalacturonides Over Time. The levels of phytoalexin-elicitor-active undecagalacturonide and dodecagalacturonide found in both *Xcm*- and mock-inoculated samples were summed. Each value represents the ratio of the sum of dp = 11 and 12 in the *Xcm*-inoculated cotyledons to the sum of dp = 11 and 12 in the mock-inoculated control collected at the same time. Values were from two replicates (± standard error). Value for the compatible interactions at 4 h was from one replicate.

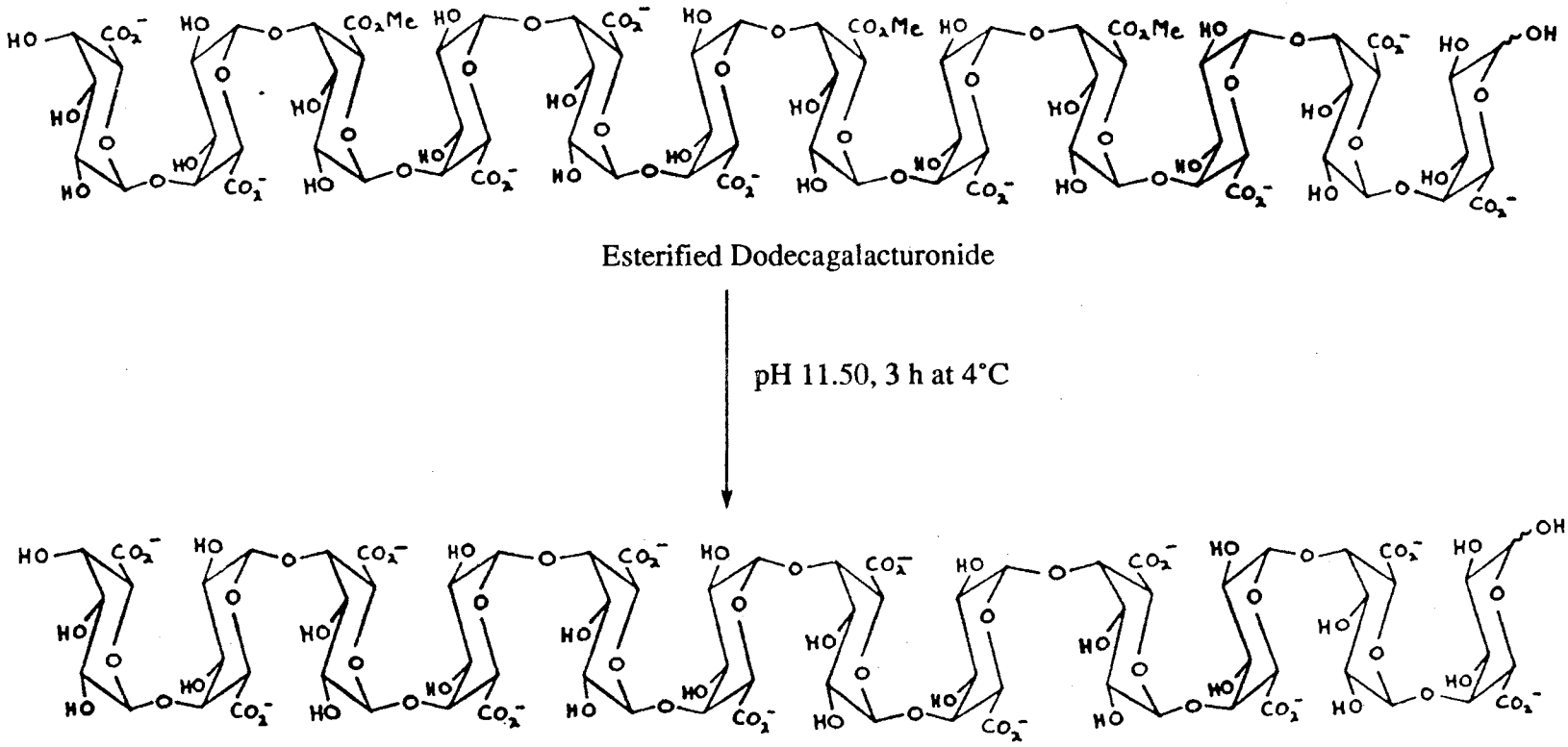


Figure 25. Reaction Depicting the Saponification of a Hypothetical Dodecagalacturonide that Contains Three Methyl Ester Groups.

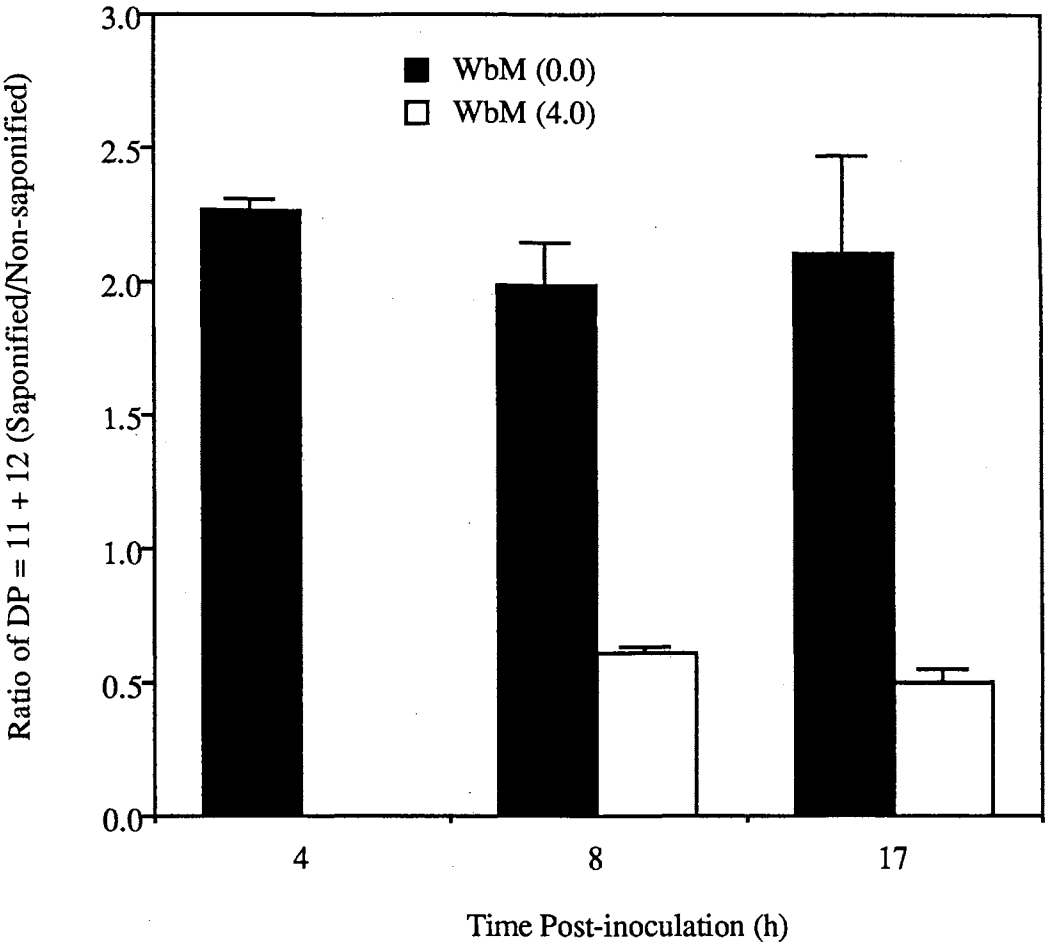


Figure 26. Results of the Saponification (Base Hydrolysis) of IWF Collected from *Xcm*-inoculated Resistant WbM (0.0) and Susceptible WbM (4.0). The purified IWF was saponified as described in the experimental section. Each bar represents ratio of the sum of the elicitor-active oligogalacturonides (*i.e.*, dp = 11 and 12) in the IWF that had undergone saponification to the sum of the elicitor-active oligogalacturonides (*i.e.*, dp = 11 and 12) from the same IWF that had not undergone saponification ± standard error.

VITA

Edgar T. Miranda

Candidate for the Degree of

Doctor of Philosophy

Thesis: ANALYSIS OF INTERCELLULAR WASH FLUIDS OF COTTON COTYLEDONS FOR THE PRESENCE OF PHYTOALEXIN-ELICITOR-ACTIVE OLIGOGALACTURONIDES

Major Field: Biochemistry

Biographical:

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Honors and Organizations: Member of Integrated Chemists of the Philippines.